IMMUNE RECONSTITUTION AFTER AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION

A Doctoral THESIS
SUBMITTED TO THE
UNIVERSIDADE NOVA DE LISBOA

BY

CRISTINA JOÃO

PhD in Medicine (Hematology)

December, 2007

"There are two objects in medical education:

to heal the sick and advance the science."

- Dr. Charles H. Mayo

I dedicate this work to my family and friends.

Table of Contents

Figures Contents

Tables Contents

Chapter VI

Chapter VII

Chapter VIII

Preface and Acknowledgments

This dissertation presents the results of my work on immune reconstitution, developed at the Mayo Clinic, Rochester, Minnesota, USA from 2002 until 2006. This work was first developed in collaboration with Dr. Jeffrey Platt and Professor Marília Cascalho and their research group, at the Transplantation Biology Division, and continued at the Hematology Division at the Mayo Clinic in Rochester, MN, USA, under the supervision of Dr. Luis Porrata and Professor Svetomir Markovic.

Along this period I relied on the close and fundamental mentorship of Prof. Maria Gomes da Silva, from Faculdade de Ciências Médicas da Universidade Nova de Lisboa. Prof. Maria Gomes da Silva has been my mentor during the Hematology Residency and Fellowship at the Instituto Português de Oncologia de Lisboa since 1999. She recognized my interest on scientific research and supported me on the decision to interrupt the Hematology Fellowship and dedicate myself fulltime to scientific research for a period of time. Prof. Maria Gomes da Silva has supported all my work and her friendship and guidance during these years have been invaluable for me.

It was through Prof. Maria Gomes da Silva that I had the opportunity to meet Professor Marília Cascalho and Dr. Jeffrey Platt, from the Transplantation Biology Division, Mayo Clinic, Rochester, Minnesota, USA and learnt about

their work. After my first visit to their laboratory, in September 2001, I applied for and was awarded with a Portuguese PhD fellowship from Fundação para a Ciência e Tecnologia and integrated Dr. Platt's team at the Mayo Clinic in June 2002.

I found the Mayo Clinic a place where biomedical research was performed with high quality standards of humanism, scientific reasoning and academic life. Mayo Clinic at Rochester, Minnesota, comprises an integrated pluridisciplinar clinic and two hospitals, staffed by more than 2,500 physicians and scientists who conduct wide-ranging research to improve patient care, while training the next generation of medical scholars. I further integrated the group of those scholars when I obtained there a Master degree in Clinical Research. It was at Mayo Clinic that I developed the studies that conducted to this dissertation.

I owe the opportunity of being trained at a very prestigious academic institution to all who allowed that training and supported this project. I deeply thank Professor António Parreira who always supported and instigated me to combine clinical work and scientific research. He is a model of a scholar leader, working to improve the scientific level of the Portuguese Medicine and Science.

I began to develop my work in immune reconstitution at the Transplantation Biology Laboratory at Mayo Clinic nested in the work of Dr. Jeffrey Platt and Professor Marília Cascalho. I started studying the influence that B

lymphocytes and immunoglobulin exert upon T lymphocyte development and function.

After two years of basic research in the area of immune reconstitution, which originated 3 publications in high impact factor scientific journals, and after starting the Mayo Clinic Master Program in Clinical Research, I began to work with Dr. Luis Porrata and Professor Svetomir Markovic at the Hematology Division of Mayo Clinic. At that time, I felt it was essential that part of my PhD studies were devoted to the clinical questions raised by my previous work.

Dr. Porrata and Professor Markovic were investigating the importance of the recovery of a normal number of lymphocytes after autologous stem cell transplantation. They previously showed that an early recovery of lymphocyte numbers after autologous stem cell transplantation in several hematological and solid neoplasms was independently associated with a prolonged survival. We hypothesized that their findings could be explained by my previous observations on T cell reconstitution under the influence of B lymphocytes. Our collaborative work soon originated two manuscripts included in this dissertation.

Professor Svetomir Markovic was the head of my Master in Clinical Research Thesis Defense Committee and directly oriented my Master thesis. Dr. Luis Porrata is my co-mentor in the PhD studies, and has co-guided my work for the last two years. A deep feeling of gratitude and friendship will always link

me to Professor Markovic and Dr. Porrata, who helped and lead me to pursue clinical scientific research after the conclusion of the first part of this work.
I thank their availability and expert discussion of scientific questions and their continuous intellectual support and alliance.

I am also sincerely grateful to Prof. António Coutinho, director of the Instituto Gulbenkian de Ciência (IGC). After returning from the United States, Professor Coutinho invited me to start a new research project as an independent junior investigator at IGC, granting me a new "research house". He has always incentivated and discussed my work and critically looked at my data.

To my colleagues and friends at the Mayo Clinic, Brenda Ogle, Gregory Brunn, Cecilia Rietz, Claudia Ferreira, Catarina Cortesão, Pedro Geraldes, Hilal Maradit-Kremers, Dianne Khurana, Vini Khurana and Xiaosheng Wu, I deeply thank all the support, friendship and intellectual discussions.

I thank with all my heart my family and close friends, who always accompanied and supported this work.

Finally, the work presented and discussed here nested already two projects that are being developed at IGC, Oeiras and at the Mayo Clinic, USA. These two projects are presented at "Future Directions" section.

As determined in the "Decreto Lei nº 388/70, artigo 8º, parágrafo 2", the results included in this thesis are already published or are being prepared to submit for publication, in the following manuscripts:

1. Ogle B., Cascalho M., **João C**., Taylor W., West J. L., Platt L. J. "Direct measurement of lymphocyte receptor diversity". ***Nucleic Acids Research*, 31:e139, 2003.**

   My contributions to this paper were to design, perform, analyse and describe the experiments related to the immunization of the mice. I wrote the part of the manuscript related with the immunization; I commented the rest of the manuscript and actively participated in writing the discussion.

2. **João C**., Ogle B., Gay-Rabinstein C., Platt J. L., Cascalho M., "B cell-dependent TCR diversification". ***Journal of Immunology*, 172:4709-4716, 2004.**

   My contributions to this paper were to design, perform, analyse and describe the majority of the experiments. I wrote the manuscript and helped to submit it.

3. **João C.,** Ogle B., Geyer S., "Immunoglobulin promotes the diversity and the function of T cells". ***European Journal of Immunology*, 36:1718-1728, 2006.**

   My contributions to this paper were to design, perform and analyse the vast majority of the experiments. I wrote the manuscript and submitted the paper.

4. **João C.**, Porrata L.F., Inwards D. J., Ansell S., Micallef I., Johnston P., Gastineau D.A., Markovic S. N., "Early lymphocyte recovery after autologous stem cell transplantation predicts superior survival in Mantle Cell Lymphoma". ***Bone Marrow Transplantation*, 37:865-871, 2006.**

   My contributions to this paper were to conceive the statistical strategy, to analyse the data set and write the manuscript.

5. **João C.**, Geyer S., Markovic S. N., Gertz M., Lacy M., Dispenzieri A., Kumar S., Hayman S., Gastineau D., Porrata L. P., "Recovery of polyclonal immunoglobulin serum levels to normal levels after autologous stem cell transplantation predicts disease free survival in patients with multiple myeloma". **In preparation, 2007.**

   My contributions to this paper were to design the analysis and the data set, collect the data, conceive the statistical analysis, analyse the data and write the manuscript.

6. **João C.**, Porrata L.F., Witzig T., Kurtin P., Micallef I., Markovic S.N., Erlichman C., Novak A., Ansell S., "Absolute lymphocyte count and CD4 count predict a superior progression-free survival in non-Hodgkin's lymphoma patients treated with Rituximab and Interleukin-12". **Submitted, 2007.**

   My contributions to this paper were to elaborate and perform the analysis and write the manuscript.

Abstract

The investigation of the web of relationships between the different elements of the immune system has proven instrumental to better understand this complex biological system. This is particularly true in the case of the interactions between B and T lymphocytes, both during cellular development and at the stage of cellular effectors functions. The understanding of the B–T cells interdependency and the possibility to manipulate this relationship may be directly applicable to situations where immunity is deficient, as is the case of cancer or immune suppression after radio and chemotherapy.

The work presented here started with the development of a novel and accurate tool to directly assess the diversity of the cellular repertoire (Chapter III). Contractions of T cell receptor diversity have been related with a deficient immune status. This method uses gene chips platforms where nucleic acids coding for lymphocyte receptors are hybridized and is based on the fact that the frequency of hybridization of nucleic acids to the oligonucleotides on a gene chip varies in direct proportion to diversity. Subsequently, and using this new method and other techniques of cell quantification I examined, in an animal model, the role that polyclonal B cells and immunoglobulin exert upon T cell development in the thymus, specifically on the acquisition of a broader repertoire diversity by the T cell receptors (Chapter IV and V). The hypothesis tested was if the presence of more diverse peptides in the thymus, namely polyclonal immunoglobulin, would induce the generation of more diverse T cells precursors. The results

obtained demonstrated that the diversity of the T cell compartment is increased by the presence of polyclonal immunoglobulin. Polyclonal immunoglobulin, and particularly the Fab fragments of the molecule, represent the most diverse self-molecules in the body and its peptides are presented by antigen presenting cells to precursor T cells in the thymus during its development. This probably contributes significantly to the generation of receptor diversity.

Furthermore, we also demonstrated that a more diverse repertoire of T lymphocytes is associated with a more effective and robust T cell immune function *in vivo*, as mice with a more diverse T cell receptors reject minor histocompatiblility discordant skin grafts faster than mice with a shrunken T cell receptor repertoire (Chapter V). We believe that a broader T cell receptor diversity allows a more efficient recognition and rejection of a higher range of external and internal aggressions. In this work it is demonstrated that a reduction of TCR diversity by thymectomy in wild type mice significantly increased survival of H-Y incompatible skin grafts, indicating decrease on T cell function. In addiction reconstitution of T-cell diversity in mice with a decreased T cell repertoire diversity with immunoglobulin Fab fragments, lead to a increase on TCR diversity and to a significantly decreased survival of the skin grafts (Chapter V). These results strongly suggest that increases on T cell repertoire diversity contribute to improvement of T cell function. Our results may have important implications on therapy and immune

reconstitution in the context of AIDS, cancer, autoimmunity and post myeloablative treatments.

Based on the previous results, we tested the clinical hypothesis that patients with haematological malignancies subjected to stem cell transplantation who recovered a robust immune system would have a better survival compared to patients who did not recover such a robust immune system. This study was undertaken by the examination of the progression and overall survival of 42 patients with mantle cell non-Hodgkin lymphoma receiving autologous hematopoietic stem cell transplantation (Chapter VI). The results obtained show that patients who recovered higher numbers of lymphocytes soon after autologous transplantation had a statistically significantly longer progression free and overall survivals. These results demonstrate the positive impact that a more robust immune system reconstitution after stem cell transplantation may have upon the survival of patients with haematological malignancies.

In a similar clinical research framework, this dissertation also includes the study of the impact of recovering normal serum levels of polyclonal immunoglobulin on the survival of patients with another B cell haematological malignancy, multiple myeloma, after autologous stem cell transplantation (Chapter VII). The relapse free survival of the 110 patients with multiple myeloma analysed was associated with their ability to recover normal serum levels of the polyclonal compartment of immunoglobulin. These results suggest again the important effect of polyclonal immunoglobulin for the (re)generation of the immune competence.

We also studied the impact of a robust immunity for the response to treatment with the antibody anti CD20, rituximab, in patients with non-Hodgkin's lymphoma (NHL) (Chapter VIII). Patients with higher absolute counts of CD4+ T lymphocytes respond better (in terms of longer progression free survival) to rituximab compared to patients with lower number of CD4+ T lymphocytes. These observations highlight again the fact that a competent immune system is required for the clinical benefit of rituximab therapy in NHL patients.

In conclusion, the work presented in this dissertation demonstrates, for the first time, that diverse B cells and polyclonal immunoglobulin promote T cell diversification in the thymus and improve T lymphocyte function. Also, it shows that in the setting of immune reconstitution, as after autologous stem cell transplantation for mantle cell lymphoma and in the setting of immune therapy for NHL, the absolute lymphocyte counts are an independent factor predicting progression free and overall survival.

These results can have an important application in the clinical practice since the majority of the current treatments for cancer are immunosuppressive and implicate a subsequent immune recovery. Also, the effects of a number of anti-neoplastic treatments, including biological agents, depend on the immune system activity. In this way, studies similar to the ones presented here, where methods to improve the immune reconstitution are examined, may prove to be instrumental for a better understanding of the immune system and to guide more efficient treatment options and the design of future clinical trials.

Resumo

O estudo da rede de inter-relações entre os diversos elementos do sistema immune tem-se mostrado um instrumento essencial para uma melhor compreensão deste complexo sistema biológico. Tal é particularmente verdade no caso das interacções entre os linfócitos B e T, quer durante o desenvolvimento celular, quer ao nível das funções celulares efectoras. A compreensão da interdependência entre linfócitos B e T e a possibilidade de manipular esta relação pode ser directamente aplicável a situações em que a imunidade está deficiente, como é o caso das doenças neoplásicas ou da imunossupressão após radio ou quimioterapia.

O trabalho apresentado nesta dissertação iniciou-se com o desenvolvimento de um novo método laboratorial para medir directamente a diversidade do reportório celular (Capítulo III). Reduções da diversidade do reportório dos receptores de células T têm sido relacionadas com um estado de imunodeficiência. O método desenvolvido utiliza "gene chips", aos quais hibridizam os ácidos nucleicos codificantes das cadeias proteicas dos receptores linfocitários. A diversidade é calculada com base na frequência de hibridização do ácido nucleico da amostra aos oligonucleótidos presentes no "gene chip". De seguida, e utilizando este novo método e outras técnicas de quantificação celular examinei, num modelo animal, o papel que as células policlonais B e a imunoglobulina exercem sobre o desenvolvimento linfocitário T no timo, especificamente na aquisição de um reportório diverso de receptores T (Capítulos IV e V). Testei, então, a hipótese de que a

presença no timo de péptidos mais diversos, como a imunoglobulna policlonal, induzisse a génese de precursores T mais diversos.

Demonstrámos que a diversidade do compartimento T é aumentado pela presença de imunoglobulina policlonal. A imunoglobulina policlonal, e particularmente os fragmentos Fab desta molécula, representam as moléculas autólogas mais diversas presentes nos organismos vertebrados. Estes péptidos são apresentados por células apresentadoras de antigénio às células precursoras T no timo, durante o desenvolvimento celular T. Tal, provavelmente, contribui para a génese da diversidade dos receptores.

Também demonstrámos que a presença de um reportório mais diverso de linfócitos T se associa a um incremento da função imunológica T *in vivo.* Uma diversidade de receptores T mais extensa parece permitir um reconhecimento e rejeição mais eficientes de um maior número de agressores internos e externos. Demonstrámos que ratinhos com receptores de células T (RCT) com maior diversidade rejeitam transplantes cutâneos discordantes para antigénios minor de histocompatibilidade mais rapidamente do que ratinhos com um menor reportório T (Capítulo V). Por outro lado, uma redução da diversidade do RCT, causada por timectomia de ratinhos de estirpes selvagens, mostrou aumentar significativamente a sobrevivência de transplantes cutâneos incompatíveis para o antigénio H-Y (antigénio minor de histocompatibilidade), indicando uma diminuição da função linfocitária T.

Além disso, a reconstituição da diversidade dos linfócitos T em ratinhos com uma diversidade de reportório T diminuída, induzida pela administração de fragmentos Fab de imunoglobulina, conduz a um aumento da diversidade dos RCT e a uma diminuição significativa da sobrevivência dos enxertos cutâneos (Capítulo V). Estes resultados sugerem que o aumento do reportório de células T contribui para uma melhoria das funções celulares T e poderão ter implicações importantes na terapêutica e reconstitutição imunológica em contexto de SIDA, neoplasias, autoimunidade e após tratamentos mieloablativos.

Baseado nos resultados anteriores, decidimos testar a hipótese clínica de que doentes com neoplasias hematológicas sujeitos a transplantação de precursores hematopoiéticos e com recuperação imunológica precoce após transplante teriam uma sobrevivência mais longa do que doentes que não recuperassem tão bem a sua imunidade.

Analisámos a sobrevivência global e sobrevivência sem doença de 42 doentes com linfoma não Hodgkin de células do manto sujeitos a transplante autólogo de precursores hematopoiéticos (Capítulo VI). Os resultados obtidos mostraram que os doentes que recuperaram contagens mais elevadas de linfócitos imediatamente após o transplante autólogo, apresentaram uma sobrevivência global e sem progressão mais longa do que doentes que não recuperaram contagens linfocitárias tão precocemente. Estes resultados demonstram o efeito positivo de uma reconstitutição imunológica robusta

após transplante de presursores hematopoiéticos, sobre a sobrevivência de doentes com neoplasias hematológicas.

Do mesmo modo, estudámos o efeito que a recuperação de níveis séricos normais de imunoglobulina policlonal tem na sobrevivência de doentes com outras neoplasias hematológicas de linfócitos B, como o mieloma múltiplo, após transplante autólogo de precursos hematopoiéticos (Capítulo VII). A sobrevivência livre de doença dos 110 doentes com mieloma múltiplo analizados está associada com a sua capacidade de recuperar níveis séricos normais do compartmento policlonal de imunoglobulina. Estes resultados pioneiros indicam a importância da imunoglobulina policlonal para a génese de competência imunológica.

Também estudámos o impacto de um sistema imunitário eficiente sobre a resposta ao tratamento com o anticorpo anti CD20, rituximab, em doentes com linfoma não Hodgkin (LNH) (Capítulo VIII). Os resultados mostram que doentes com valores mais elevados de linfócitos T CD4+ respondem melhor (em termos de maior sobrevida livre de doença) ao rituximab, do que doentes com valores mais baixos. Estas observações ilustram a necessidade de um sistema imunitário competente para o benefício clínico da terapêutica com rituximab em doentes com LNH.

Em conclusão, o trabalho apresentado nesta dissertação demonstra que as células B e a imunoglobulina policlonal promovem a diversidade das células T no timo e melhoram a função linfocitária T periférica.

Concomitantemente, também demonstrámos que, no contexto de reconstituição imune, por exemplo, após transplante autólogo de precursores hematopoiéticos em doentes com linfomas de células do manto, o número absoluto de linfócitos é uma factor independente da sobrevivência. Os resultados demonstram, também, a importância dos valores de linfocitos T na resposta ao tratamento com rituximab no caso de doentes com LNH. O mesmo princípio se prova pelo facto de que doentes com mieloma múltiplo sujeitos a transplante autólogo de precursores hematopoiéticos que recuperam valores normais séricos de imunoglobulinas policlonais, terem melhores taxas de resposta em comparação com doentes que não recuperam valores normais de imunoglobulinas policlonais. Estes resultados podem ter importantes aplicações na prática clínica dado que a maioria dos tratamentos de doenças neoplásicas implica imunossupressão e, subsequente, recuperação imunológica. Estes estudos podem ser um instrumento fundamental para uma melhor compreensão do sistema imune e guiar uma escolha mais eficiente de opções terapêuticas bem como contribuir para a concepção de futuros estudos clínicos.

Résumé

L'étude du réseau d'interrelations entre les divers éléments du système immune s'est montré un instrument essentiel pour une meilleure compréhension de ce système biologique complexe. C'est particulièrement vrai dans le cas des interactions entre les lymphocytes B et T, soit pendant le développement cellulaire soit à l'étape des fonctions cellulaires d'effecteurs. La compréhension de l'interdépendance entre des lymphocytes B et T et la possibilité de manipuler cette relation peuvent être directement applicables à des situations où l'immunité est déficiente, comme c'est le cas du cancer ou d'immunosuppression après la radiothérapie et la chimiothérapie

Le travail présenté dans cette dissertation a commencé avec le développement d'un nouveau et précis méthode en laboratoire pour mesurer directement la diversité du répertoire cellulaire (Chapitre III). Des contractions dans la diversité du répertoire des récepteurs de cellules T ont été reliées avec un état d'insuffisance immunologique. Cette méthode utilise "gene chips", où les acides nucléiques codificateurs des chaînes protéiques des récepteurs lymphocytaires sont hybridés. La diversité est calculée sur la fréquence d'hybridation de l'acide nucléique de l'échantillon aux oligonucléotides présents dans le "gene chip". En suite, et en utilisant cette nouvelle méthode et d'autres techniques de quantification cellulaire, j'ai examiné, dans un modèle animal, le rôle que les cellules polyclonales B et l'immunoglobuline exercent sur le développement lymphocytaire T dans le thymus, spécifiquement dans l'acquisition d'un répertoire divers de

récepteurs T (Chapitres IV et V). L'hypothèse évalué était si la présence dans le thymus de peptides plus diverses, comme l'immunoglobuline polyclonale, induirait la génération de précurseurs T plus divers.

Les résultats obtenus ont démontré que la diversité du compartiment de cellules T est augmentée par la présence d'immunoglobuline polyclonale. L'immunoglobuline polyclonale, et particulièrement les fragments Fab de cette molécule, représente les auto-molécules les plus divers dans les organismes vertébrés. Ces peptides sont présentés par des cellules présentateurs d'antigène à cellules précurseur T dans le thymus, pendant le développement cellulaire T. Ceci probablement contribue significativement, à la génération de la diversité des récepteurs.

Nous avons aussi démontré que la présence d'un répertoire plus divers de lymphocytes T est associée avec une fonction immunologique T plus efficace *in vivo* puisque des petits rat avec des récepteurs de cellules T (RCT) plus diverses rejettent des transplants cutanés discordants pour antigènes *minor* de histocompatibilité plus rapidement que des rats avec un répertoire T moindre (Chapitre V).

Une diversité de récepteurs T plus étendue semble permettre une reconnaissance et un rejet plus efficaces pour un plus grand nombre d'agresseurs internes et externes. Dans cette étude, il est démontré qu'une réduction de la diversité de RCT, par tymectomie des rats sauvages, augmente significativement la survie de greffes cutanés incompatibles pour l'antigène H-Y (antigène *minor* d'histocompatibilité), indiquant une

diminution de la fonction lymphocytaire T. D'autre part, la reconstitution de la diversité des lymphocytes T dans des rats avec une diversité diminuée du répertoire T, réussie par l'administration de fragments Fab d'immunoglobuline, conduit à une augmentation de la diversité de RCT et à une diminution significative de la survie des greffes cutanées (Chapitre V). Ces résultats suggèrent que l'augmentation de la diversité du répertoire de cellules T contribue à une amélioration des fonctions cellulaires T. Ces résultats pourront avoir des implications importantes dans la thérapeutique et la reconstitution immunologique dans le contexte de SIDA, néoplasies, auto-immunité et après traitements myéloablatives.

Nous d'étudions l'hypothèse clinique de que, chez des malades avec des néoplasies hématologiques sujets à la transplantation de précurseurs hématopoïétiques qui récupéraient un système immunologique plus robuste précocement après transplant, auraient plus longues survies en comparaison avec des malades qui ne récupéraient pas aussi bien leur immunité. Nous conduisons cette étude en analysant la survie globale et la survie libre de maladie de 42 malades avec lymphome non Hodgkin de cellules du manteau sujets à transplant autologue de précurseurs hématopoïétiques (Chapitre VI). La survie de ces malades a été analysée conformément à compte des lymphocytes récupérés au 15éme jour après la greffe. Les résultats obtenus ont montré que les malades qui ont récupéré des valeurs plus élevées de lymphocytes immédiatement après le transplant autologue, ont présenté une survie globale et une survie libre de progression, statistiquement plus longue

que malades que n'ont pas récupéré de comptages lymphocytaire aussi élevés. Ces résultats démontrent l'effet positif de la reconstitution immunologique robuste, après transplant de précurseurs hématopoïétiques pour la survie des malades avec des néoplasies hématologiques.

Dans un cadre de recherche clinique, cette dissertation inclut aussi l'étude de l'effet que la récupération de niveaux sériques normaux d'immunoglobuline polyclonale sur la survie de malades avec autres néoplasies hématologiques de lymphocyte B, comme le myélome multiple, après transplant autologue de précurseurs hématopoïétiques (Chapitre VII). La survie libre de maladie chez 110 malades avec myélome multiple a été associée à la capacité de récupération des niveaux sériques normaux du compartiment polyclonale d'immunoglobuline. Ces résultats indiquent, à nouveau, l'importance de l'immunoglobuline polyclonale pour la génération de la compétence immunologique.

Nous avons aussi étudié l'impact que un système immunitaire robuste aura dans la réponse au traitement avec l'anticorps anti CD20, rituximab, chez des malades avec lymphome non Hodgkin (LNH) (Chapitre VIII). Ces résultats, ici présentés, montrent que les malades avec des valeurs plus élevées de lymphocytes T CD4 +, répondent mieux à rituximab, en comparaison avec des malades avec basses valeurs de lymphocytes T CD4+. Ces observations illustrent que un système immunitaire compétent est nécessaire pour le bénéfice clinique de la thérapeutique avec rituximab des malades avec LNH.

En conclusion, le travail présenté dans cette dissertation démontre que les cellules B et l'immunoglobuline polyclonale promeuvent la diversité de cellules T dans le thymus et améliorent la fonction lymphocytaire T périphérique.

Il montre aussi que, dans le contexte de reconstitution immune, par exemple après un greffe autologue de précurseurs hématopoïétiques chez des malades avec des lymphomes de cellules du manteau, le nombre absolu de lymphocytes est un facteur prédictif indépendant de la survie libre de progression de la maladie et de la survie globale. Les résultats de ce travail démontrent, aussi, l'importance que la robustesse du système immunitaire dans la réponse au traitement avec rituximab chez les malades avec LNH. En effet, des malades avec des nombre plus élevées de lymphocytes T CD4+ présentent des taux de réponse plus haute quand comparés avec des malades avec des valeurs plus basses. Le même principe se prouve chez les malades avec myélome multiple sujets à transplant autologue de précurseurs hématopoïétiques qui récupèrent des valeurs sériques normales d'immunoglobulines polyclonales. Ils ont des meilleurs taux de réponse en comparaison avec les malades qui ne récupèrent pas de valeurs normales. Ces résultats peuvent avoir importantes applications dans la pratique clinique puisque la majorité des traitements de maladies néoplasiques implique immunosuppression et, ultérieure, récupération immunologique. Aussi, les effets d'un grand nombre de traitements antinéoplasiques, agents biologiques inclus, dépendent de l'activité du système immune. C’études

peuvent être un instrument fondamental pour une meilleure compréhension du système immunitaire, et guide un efficace choix d'options thérapeutiques et aide un le dessin de futures études cliniques.

Chapter I

General Introduction

The role of B cells and immunoglobulin in T cell development

The immune system is one of the most complex biological systems in vertebrate animals and it can be systematically divided in innate and adaptive. The innate immunity includes the components of the immune system that do not recombine its receptors to produce a diverse repertoire. The basic protective strategy of an innate immune system allows the organism to constitutively produce generic, germ line-encoded receptors. These receptors recognize conserved patterns on different classes of pathogens and trigger an inflammatory response that limits pathogen invasion [1]. The innate compartment includes cellular elements (natural killer cells, macrophages and inflammatory cells) and all the physical barriers protecting the body.

On the other way, the adaptive immune system includes cells that clonally generate a diverse repertoire. In this compartment, each cell bears a unique antigen receptor. The adaptive immune system is present in all jawed vertebrates that assemble their antigen-receptor genes through recombinatorial rearrangement of different immunoglobulin or T cell receptor gene segments [2]. Lymphocytes, the specialized cell types of the adaptive immune system, use their cellular receptors to recognize antigenic configurations of specific pathogens and then respond to the antigen by

clonal amplification, cellular differentiation, and production of antibodies with the same antigen binding specificity [3].

Two major lineages of lymphocytes that can specifically recognize and respond to antigenic determinants of potentially hazardous pathogens are generated in the thymus (the T lymphocytes) and the bone marrow or the avian bursa of Fabricius (the B lymphocytes) [4]. T and B cell compartments are thought to develop separately. The concept that lymphoid compartments develop separately derives from observations made nearly fifty years ago on the ablation of lymphoid organs. Removing the Bursa of Fabricius, an out-pouching of the gut of birds, by either hormonal or surgical means during the first days of life, renders a bird incapable of producing antibodies but able to reject an allograft [5]. Removing the thymus of a mouse within 24 h of birth abrogates the ability to reject an allograft but leaves the mouse able to produce antibodies [6, 7]. These classical experiments established that there were "at least two basic levels of immune responses related functionally to different primary lymphoid organs", the thymus and the bursa of Fabricius [8]. One level of responses requires thymus integrity and concerns allograft rejection and graft-versus host disease while the other, requiring the bursa, is necessary for antibody production [4, 8, 9]. With the subsequent discovery that lymphoid cells develop in the Bursa of Fabricius and in the thymus, the former were referred to as "B" cells and the later as "T" cells [10].

Several subtypes of T cells have been considered: helper T cells (T cells expressing surface CD4 molecules), cytotoxic T cells (T cells expressing surface CD8 glycoprotein), memory T cells, regulatory T, NKT cells and $\gamma\delta$ T cells. All these cell types have different and specifically recognized functions.

Helper T cells could be seen as the major driving force and the main regulators of the immune defense. Their primary task is to activate B cells and cytotoxic T cells [11]. However, the helper T cells themselves must be activated. This occurs by means of action of antigen presenting cells, namely dendritic cells, macrophages or B cells. Once activated, helper T cells proliferate and produce cytokines that activate B and T cells as well as other immune cellular elements.

The cytotoxic CD8+ T cells are specialized in recognizing and killing cells of the body infected by viruses and bacteria, and also cancer cells.

Memory T cells are a subset of antigen-specific T cells that persist for long periods after an infection has resolved and rapidly expand if they are exposed again to the same antigens. Memory cells may be either CD4+ or CD8+, and can be functionally divided in central and effector memory T cells [12, 13].

Regulatory T cells (T regs) are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell mediated immunity and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus [14]. Naturally occurring T regs (phenotypically characterized

as $CD4^+CD25^+FoxP3^+$ T cells) arise in the thymus, whereas the adaptive T regs may also originate during a normal immune response [15, 16].

Natural Killer T cells (NKT cells) bridge the adaptive with the innate immune system. They do not use major histocompatibility complex (MHC) molecules to recognize peptide antigens but employ CD1 to recognize glycolipid antigen [17].

$\gamma\delta$ T cells represent a small subset of T cells (around 5%) that express a surface TCR made up of one γ- and one δ-chain and are mainly found in the gut. These T cells are not MHC restricted and seem to be able to recognize whole proteins rather than peptides [18].

A previous study, using mice engineered to express monoclonal T cells with or without monoclonal B cells, found that mice producing both T cells and B cells (called monoclonal B-T mice or MBT mice) have 2-fold more thymocytes and 35-fold more peripheral CD4+ T cells than mice that make only T cells (called monoclonal T cell mice or MT mice) [19]. One possible explanation for these observations is that B cells might in some way promote the development of T cells. This work launched further investigations on T-B relationships during cell development.

To understand how B cells might contribute to the development of T cells in the thymus, it is useful to consider how the thymus is thought

to promote T cell development (reviewed by Hergen Spits in [20]). The process of T cell development includes not only the generation of thymocytes but also their maturation to promote the ability to recognize self-MHC without autoimmunity. TCR genes recombine to yield approximately 10^{15} different TCRs [21]. From these TCRs, only around 10^{8} to 10^{11} are selected in humans [22, 23] and around 10^{6} in mice [24]. This process is called positive selection and allows the survival of the thymocytes in which TCRs recognize self-peptides presented by self-MHC molecules with an intermediate affinity. In contrast, thymocytes with TCRs that recognize self-peptides with a high affinity are negatively selected and die by apoptosis, protecting the organism from autoimmunity. Also, thymocytes which TCRs not recognizing self peptides-MHC complexes or recognizing them with a very low affinity die by lack of stimulus via the TCR (neglect). The fact that each T cell precursor, through its TCR, can only recognize self-peptides presented by self-MHC molecules is named "restriction" [25, 26]. These two processes (selection and restriction) warrant that only T cells that recognize the self and are not highly self-reactive are "selected", and survive to egress to the periphery.

The process of T cell development is generally divided in phases defined on the basis of different surface molecules expressed by the thymocytes. Also, each phase is characterized by distinct molecular events. The most immature thymocytes comprise a small cellular population in the thymus that

do not express either CD4 or CD8 and are called double negative (DN). In this phase, thymocytes rearrange the TCR β chain and undergo proliferation and expansion. After rearranging the β chain, through a stochastic process of VDJ gene segment recombination, thymocytes begin to express membrane CD4 and CD8 molecules, becoming double positives (DP) (see Figure 1).

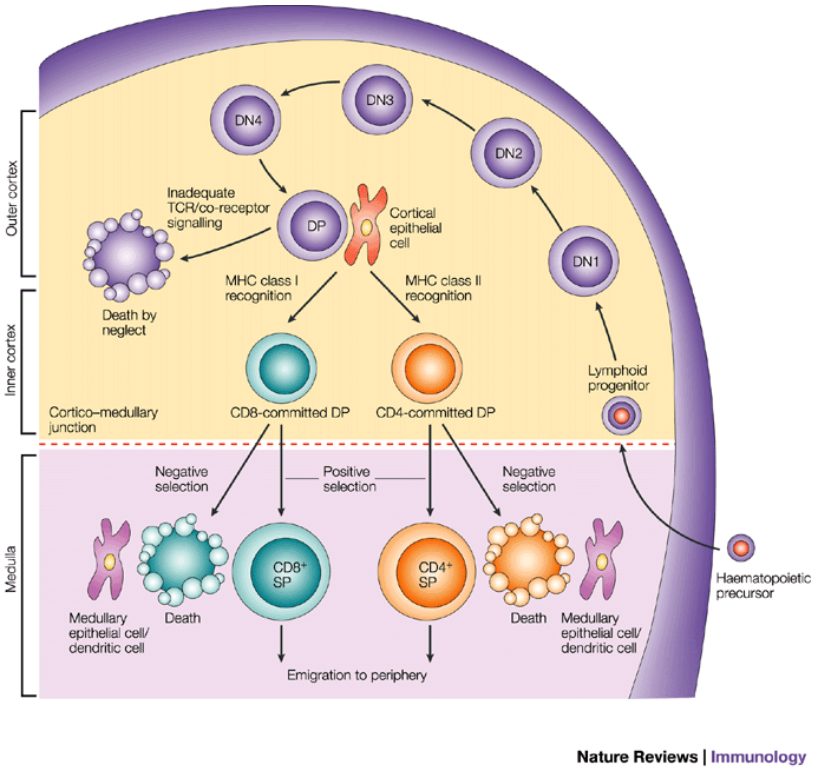


Figure 1 – T cells development in the thymus.
The T cells precursors enter the thymus by the cortico-medullar junction and, in contact with thymic epithelial cells, undergo processes of selection and restriction in order to survive. Before leaving the thymus as mature/naïve T cells, the T cells' precursors rearrange the DNA encoding the TCR chains and acquire different membrane markers.
(Adapted from Hergen Spits, Nature Reviews Immunology, 2002 [20])

Arstila el al. calculated that the β chain rearrangements can originate at least 10^6 different β chains and the α chain rearrangement can originate a repertoire of ~0.5×10^6 different chains [22]. These different α and β chains pair together to form a heterodimer; it is estimated that one β chain pairs, on average, with 25 different α chains, generating a final T cell receptor repertoire which diversity was predicted to be around 10^7 in humans [22].
Since only one percent of thymocytes that begin development reach maturity, the critical step in modelling the T cell repertoire must be positive selection. Positive selection must select T cells capable of recognizing self-MHC with intermediate affinity and these selected T cells also must be capable of recognizing "foreign" peptides associated with that MHC. Thus, as a consequence of T cell development, T cells should be able to interact with peptides from the range of bacteria, viruses and toxins that threaten health. It is generally believed that the combined diversity of the T cell repertoire and positive selection process will enable a response towards any pathogenic "challenge".

Several hypotheses can be formulated to address how this functional T cell repertoire is generated. One hypothesis is that different proteins produced within the thymus and, presented by self-MHC, originate diversity. However, only a limited number of different peptides are found in the thymus and many of them seem to contribute to negative selection [27]. Another

hypothesis is that TCR cross-react with many peptides. However, too much cross reactivity between TCR molecules would promote autoimmunity. Thus, although it happens, cross reactivity *per se* should be limited.

A third hypothesis, and the one favoured in this work, is that the immune system itself generates diverse peptides that select for diversity. In this setting, potential sources of diversity are B cell receptors and serum immunoglobulins. B cell receptors and circulating immunoglobulins contain variable regions as diverse as the TCR [21] and we hypothesized these proteins help selecting T cell receptors. This hypothesis is tested in the work presented in this dissertation, in particular at Chapter IV and V.

Immune Reconstitution after autologous hematopoietic stem cells transplantation and after treatment with rituximab. The cases of Non-Hodgkin Lymphomas and Multiple Myeloma

There are several neoplastic diseases where high-dose treatments have proved to be more effective than conventional dose therapies in what concerns response rate, progression free survival and overall survival. This is the case of mantle cell lymphoma and multiple myeloma that were studied in Chapter VI, VII, and VIII. When extremely high doses of chemotherapy and/or radiotherapy are administered, fatal hematopoietic toxicity needs to be prevented by the infusion of hematopoietic stem cells, either autologous or allogeneic. Allogeneic hematopoietic stem cells have the advantage of the immunologic effect of graft versus tumor, through the recognition of different HLA class I and II antigens and co-stimulatory molecules on tumor cells that lead to the generation of alloreactive T cells [28] **.**

Autologous HSCT involves harvest and storage of patients' HSC followed by high-dose chemotherapy aiming to eradicate the patient's malignant cell population. This occurs at the cost of also eliminating the patient's hematopoietic stem cells and is followed by rescue through the re-infusion of the patient's stored stem cells. Autologous transplants have the advantage of a lower risk of graft rejection and infection, since the immune recovery is

faster. There is no opportunity for graft-versus-host disease, since the donor and recipient are the same.

Allogeneic HSC donors must have major histocompatibility antigens that, at least partially, match the recipient's. Even when a good match is found, the recipient will require high levels of immunosuppression to prevent graft-versus-host disease. Allogeneic transplant donors may be related (sibling) or unrelated volunteers. Allogeneic stem cell transplantation aims, not only to the rescue of hematopoiesis, but also to improve the immunological effect graft versus tumor, as a means of promoting engraftment, accelerating immune recovery and strengthening the anti-neoplastic effects.

High dose chemotherapy followed by autologous hematopoietic stem cell transplantation (ASCT) has been associated with prolonged disease-free survival in numerous malignancies including relapsed non-Hodgkin's lymphoma [29], acute myelogenous leukemia [30], multiple myeloma [31] and a number of solid tumors [32, 33]. Approximately 105,000 autologous hematopoietic stem cell transplants were performed until 2004 for malignant diseases based on several American registries published by the Center of International Blood and Marrow Transplant Research [34]. Of those, 27,419 were performed for NHL and 20,423 for plasma cell disorders [34].

Non-Hodgkin lymphoma (NHL) is the most frequent hematological tumor, representing 4% of all neoplasms; for unclear reasons, its incidence has been constantly rising in the Western world. The WHO classification of NHL includes more than 30 biologically and clinically distinct entities. B cell NHL,

that comprise 90% of those entities, includes indolent (mostly follicular, lymphocytic, marginal zone and lymphoplasmocytic) and aggressive (mostly diffuse large cell, mantle cell, Burkitt, and lymphoblastic) histological forms [35]. Indolent diseases are generally incurable but follow a prolonged course, with frequent need for therapy, while aggressive forms can be cured in a significant proportion but have a global shorter survival. Currently, treatment for most forms of B-cell NHL at diagnosis and/or at relapse includes chemotherapy associated with the anti B-cell monoclonal antibody Rituximab, a chimeric murine/human monoclonal antibody directed to the CD20 antigen that has been shown to induce complement- and antibody-dependent cell mediated lysis, as well as apoptosis of tumor cells and sensitization to the cytotoxic effects of anti-neoplastic drugs [36]. Another treatment option for Non Hodgkin lymphomas is high dose chemotherapy followed by hematopoietic stem cells transplant (HSCT), autologous or allogeneic. This is usually an option in the case of aggressive non-Hodgkin lymphomas, as it is the case of diffuse large B cell and mantle cell lymphomas.

Multiple myeloma is the second most prevalent hematological cancer and represents approximately 1% of all cancers and 2% of all cancer deaths [37]. Treatment of multiple myeloma is currently tailored to each patient according to several patient-, disease- and economical-related factors [38] and include chemotherapy, radiation, biological treatments and ubiquitin-proteasome pathway inhibitors [39, 40]. In the case of multiple myeloma, high dose chemotherapy followed by autologous hematopoietic stem cell transplant is

the treatment of choice for the fit and young patients [31, 41, 42]. The use of allogeneic stem cell transplants to exploit the effect of graft versus myeloma effect has been recently shown effective, as survival of patients with newly diagnosed myeloma is superior among recipients of a hematopoietic stem-cell autograft followed by a stem-cell allograft from an HLA-identical sibling compared to recipients of tandem stem-cell autografts [42].

Even so, the clinical efficacy of ASCT is limited by delayed immune recovery, resulting in infectious complications and probably contributing to high tumor relapse rates (40 to 70%) [43-45].

Besides autologous stem cell transplantation many other treatment options exist to treat hematological malignancies. These options include chemotherapy, radiotherapy, radioimmunotherapy and biological agents such as antibodies, intracellular molecules inhibitors and immunomodulators.

As previously mentioned, for the most common subtypes of non-Hodgkin lymphomas, treatment at diagnosis and/or at relapse includes poly-chemotherapy regimens associated with the anti B-cell monoclonal antibody, Rituximab [46, 47].

Rituximab is a chimeric murine/human monoclonal antibody directed to the CD20 antigen that has been shown to induce complement- and antibody-dependent cell mediated lysis, as well as apoptosis of tumor cells and sensitization to the cytotoxic effects of anti-neoplastic drugs [48].

As a consequence, circulating B cells remain undetectable for at least 6 months after treatment and recover to normal levels between 6 and 9 months later [49, 50]. The homeostatic and clinical consequences of these changes are not yet clear.

Several ways to simultaneously increase the activity of rituximab and to stimulate immunity have been tried exploring the role of cytokines and growth factors like IL-2, IL-4, IL-12, IL-15, TNF-α, G-CSF, GM-CSF [48, 51-55] [56, 57]. These molecules act on T and B cells, macrophages and NK cells activating its proliferation and activity.

The effects of some of these immunomodulators are outlined in Figure 2 and listed in Table 1.

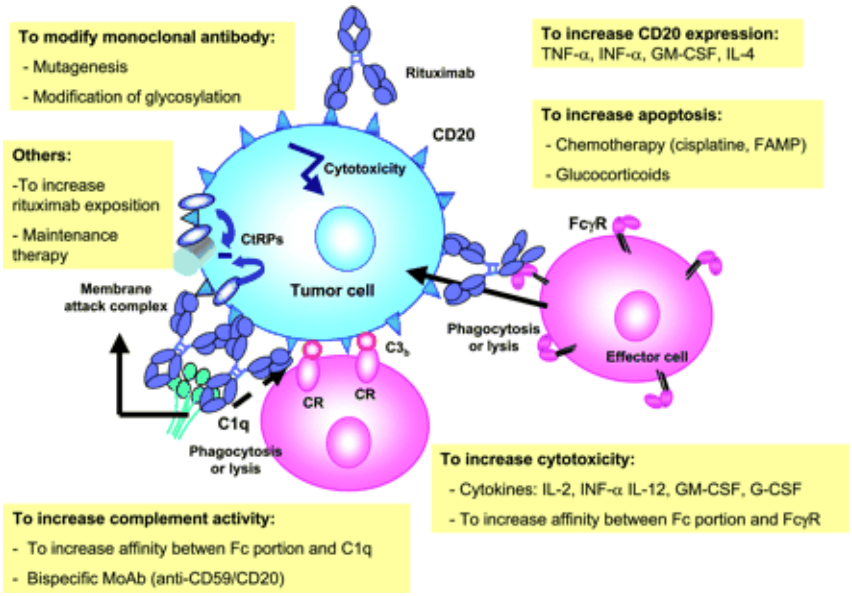


Figure 2 - **Mechanisms of action of rituximab and ways to increase its clinical efficacy.** FAMP indicates fludarabine monophosphate; CR, complement receptor. [Adapted from Carton G et al, 2004 [50].

Table 1 – Cytokines and growth factors exploited to increase patients immunity

Molecule	Effect
IL-2 [48, 55] [52]	• Increases ADCC by NK cells • Promotes activation and expansion of NK cells • Increases cytotoxicity by macrophages • Induces monocytes' production of cytokines and growth factors and expression of its receptors on monocytes; also promotes anti microbicidal and anti tumoricidal activity of monocytes. • Promotes activation and proliferation of T cells • Facilitate proliferation of B cells producing IgG1 immunoglobulins
IL-12 [55, 58]	• Increases ADCC by NK cells • Induces the secretion of IFN g by T and NK cells • Promote the development of Th1 helper T cells • Inhibits angiogenesis • Promotes antimicrobial responses to intracellular pathogens
IL-15 [57]	• Stimulate lymphocyte proliferation and activation • Promote the development and survival of NK cells, NKT cells, and intraepithelial lymphocytes
TNF-α [54] [53]	• Stimulate T lymphocyte cytotoxicity • Stimulate NK activity
G-CSF and GM-CSF [59, 60]	• Induce monocyte differentiation • Enhances the cytotoxicity of neutrophils through ADCC • Increasing neutrophil counts

As previously explained, the ability of the immune system to cope with diverse and fast evolving pathogens is achieved by the development of a vast and diverse repertoire of antigen receptors in lymphocytes that can potentially recognize any possible foreign antigens. Since B cell NHL are neoplasms of immune system, this function is usually altered. Furthermore, the majority of treatments options *per se* alter the dynamics of the immune system and cause a profound impact, in general decreasing the immunity. In fact, NHL patients frequently demonstrate clinical and biological signs of immune suppression before treatment. Chemotherapy alone strongly contributes to lymphocytopenia, significantly depleting circulating T cells, which recovery depends on multiple factors, including the intensity of the regimens used.

Several studies described the kinetics of the immune reconstitution after ASCT for hematological and other neoplasms, including the comparison of immunological reconstitution after transplant of autologous CD34+ peripheral blood progenitor cells (PBPC) and autologous unselected PBPC [61-65]. It is generally accepted that NK cells are the first population appearing within the first 2 months after ASCT. B cells appear afterwards and a low CD4+/CD8+ ratio is maintained during at least the first year post-transplant, caused by a persistent increase of CD8+ and a constant reduction of CD4+ lymphocytes.

This finding, in part, explains the patients' susceptibility to infections for a prolonged period of time post-transplant.

The reconstitution of the immune system after myelotoxic therapies has been mostly studied in the setting of hematopoietic transplantation. It may be analyzed from two distinct points of view: (1) the numerical recovery of cellular elements; and (2) the functional recovery of cellular interactions. Following hematopoietic stem cells transplantation there is a relatively rapid reappearance of hematopoietic and lymphoid cells. However, functional recovery of immune cells occurs at a much slower rate (Table 2). Complete reconstitution of the normal humoral and cellular (T and B cell) immunity may be delayed beyond one year following transplantation or, in certain settings, it may never fully recover, specially in what concerns the diversity of the T and B cell repertoire [66-71].

On the other hand serum immunoglobulin levels recovery after ASCT, which has been mainly characterized in children, seems to start with the recovery of normal levels of IgM at 6 months, followed by IgG. Only 2 to 3 years later do the IgA levels reach the normal range [72, 73].

Table 2 - Reconstitution of B and T cell numbers and function after ASCT (adapted from [74])

B-cell Reconstitution Post-ASCT		**T-cell Reconstitution Post-ASCT**	
Circulating B cells	Low from 3 to 18 months	**Circulating T cells**	
Serum		**CD4**	Low for years
IgM	Low for up to 6 months	**CD8**	Low for 3 to 18 months
IgG	Low from 12 to 18 months	**T-cell proliferation**	Low for 3 months to 5 years
IgA	Low for up to 36 months	**Cytokine production**	Low for 6 months to 5 years
***In vivo* antibody response:**		**Response to IL-2**	Low for 7 months to 5 years
T dependent antigen	Low for months to years	**Cytotoxic T cells**	Low for 2 months to 5 years
T independent antigen	Low for years		
***In vitro* B cell responses to polyclonal stimulator**	Low for months to years		

The reconstitution of NK cells has been extensively studied (reviewed by Porrata et al [74]). This compartment recovers normal absolute and relative numbers within 1 month after autologous transplantation. The reconstituted NK population seems to be fully functional in terms of gamma-IFN-secreting

activated killer cells and cytotoxicity [75-78].

The study of immune reconstitution after treatment with rituximab is limited to a few very recent published studies. Those studies report that, after treatment with the monoclonal antibody and during the reconstitution phase (3 to 15 months), the majority of the peripheral blood B cells have both an immature, naïve phenotype (CD38hiCD27-CD24hiIgD-/+) and function, being less responsive to proliferative stimuli and more prone to apoptosis when in culture [49, 50, 79, 80]. Studies of the impact on T cell immunity after treatment with rituximab are very scarce in the literature. It was recently published that a significant increase in activated CD4+ and CD8+ T cells, as well as CD25brightFOXP3+ regulatory cells, happens after treatment with rituximab [81].

Previous studies of our group at the Mayo Clinic have shown that early recovery of lymphocytes post-ASCT is critical to the clinical outcome (disease free and overall survival) in several hematological and non-hematological malignancies [82-85]. In agreement with others [86, 87], the analysis of the recovery of lymphocyte numbers at day 15 post-transplant provided evidence that timely host immune system recovery may impact on human tumor cell biology. Therefore, the therapeutic goal in ASCT should be to maximize hematopoietic engraftment and to improve immunological recovery. Effective methods that allow an accelerated immune reconstitution post-ASCT are urgently needed, as they may significantly modify the clinical outcomes of treated patients.

The possibility of manipulating the immune system with IvIg infusion

The therapeutic applications of human immunoglobulins have been investigated for decades. The largest clinical impact of intravenous polyclonal immunoglobulin therapy (IVIG) probably occurrs in the setting of immunodeficiency [88-90], autoimmunity and inflammation [91, 92] and infectious disorders [93, 94] [95]. Its rationale was based on studies of the mechanisms of action of the molecule, that justified these applications [96, 97].

The immunomodulatory effects of immunoglobulins are also related to several well established actions, including the modulation of the expression and function of Fc receptors, the interference with complement activation and cytokine networks, the provision of anti-idiotypic antibodies and the modification of the activation, differentiation and effector functions of T and B lymphocytes cells and dendritic cells [91, 98].

Immunoglobulins are a product of B cells. There are several other mechanisms by which B cells may modify T cell functions, besides the direct effects of immunoglobulin. Those include B cell antigen presentation [99, 100], the production of antibodies that may enhance [101] or depress [102] cellular immunity, B cell dependent development of follicular dendritic cells in

peripheral lymphoid organs [103], and negative effects on regulatory T cells [104].

Regarding the effect of B cells on lymphoid organogenesis, it is recognized that expression of CXCL13, a B cell chemokine, can generate organized lymphoid tissue including B cells and T cells [105]. B cell expression of LT$\alpha 1\beta 2$ contributes to the development and migration of follicular dendritic cells [106, 107]. B cell expression of LT$\alpha 1\beta 2$ is also necessary for the development of T cell zones in the spleen [108]. In the same way, differentiation of the "follicular-associated epithelium" in gut associated lymphoid tissue requires the presence of B cells [109].

It has been recently suggested that B cells have a role on the regulation of regulatory T cells. Some reports refer a positive effect of B cells via B7 on stimulating regulatory T cells [110], while others point to a negative effect of the B cell population, which may block regulatory T cells effect [104]. This is a novel field of research deserving further investigation.

The influence of peripheral compartment of B cells and immunoglobulin is also noticed in increasing peripheral TCR stimulation, which may be critical for maintenance of the naïve T cell population [111, 112]. These signals keep T cells metabolically active and thus contribute to avoid passive death. This mechanism may allow the survival of naïve T cells and at the same time the maintenance of a diverse repertoire of T cells. The reception of survival

signals through immune receptors seems to be a general principle in the biology of naïve lymphocytes involved in adaptive immunity [113]. The question of whether B cells are needed to initiate and maintain memory CD4+ T cell responses have been studied by several investigators [110, 114-116]. Those studies strongly suggest that, by promoting the initial expansion of activated antigen specific T cells, B cells have an essential role in determining the size and maintenance of the memory T cell pool and, also that, this B cell-dependent enhancement of T cell memory response may be independent of B cell antigen presentation.

Measurement of T cell receptor diversity

T and B cells generate diverse antigen-specific receptors through gene recombination; both cell repertoires are shaped during their development by recognizing self-antigens. Several estimations of the T and B cells repertoire diversity have been accomplished using various available methods. However, the different methodologies have their specific limitations, varying with each technical tool applied.

The availability of an accurate instrument to measure the diversity of the T cell repertoire is essential to examine the complexity of the immune system, either to understand its ability to recognize a diverse range of antigen determinants or to assess its limitation in the setting of lymphopenia and other situations of repertoire contractions.

Also, the availability of an accurate and direct measurement of T cell receptor repertoire is crucial for the comparison of different T cell receptor population's repertoires and the testing of different approaches to improve T cell development, in particular the generation of TCR diversity.

The more commonly used methods to measure cellular diversity are insufficient to directly assess the entire repertoire of T cell receptors, since they are based on the analysis of only a subset of this repertoire. The most widely used method to assess repertoire diversity is spectratyping analysis (also known as immunoscope) and is based on the analysis of the distribution

of the size spectrum of the TCR chain CDR3 lengths for each V family [117, 118]. One drawback of spectratyping is the fact that it is based on electrophoretic separation of the amplified lymphocyte receptor V family PCR products according to the junctional (J) sequences. Diversity is inferred from the number and electrophoretic separation patterns of amplified and re-annealed V region PCR products. Spectratyping effectively detects clonal expansion within a V family. However, since several thousand V-J family combinations for lymphocyte receptors exist, the totality of V-J combinations cannot be routinely analyzed. Several mathematical improvements have been developed for the analysis of this type of data. In fact, improvements in the integration of the parameters' read out from the sequencer were achieved [119-121]. However, all the analysis are, ultimately, based on a finite set of the entire possible repertoire and not entirely quantitative.

Other PCR-based methods, very laborious and with limited sensitivity, were developed based on the analysis of DNA encoding lymphocyte receptors by PCR amplification using constant region (C) and V-family specific primers [122]. A more recent PCR-based technique to assess DNA diversity is AmpliCot analysis, which uses DNA hybridization kinetics to assess diversity of PCR products [123]. The use of PCR methodology for the study of T cell repertoire diversity with primers specific for C and V families of the TCR chains does not differentiate among individual clones of the same family and may fail to detect balanced narrowing or expansion of the repertoire. In the case of AmpliCot analysis, the fact that this estimation of cellular

diversity is based on Cot analysis (a method based on the principle that the time required for a DNA sample to re-anneal is related to the sequence complexity of the sample) and PCR, may lead to serious decreases in specificity due to possible erroneous amplifications of impure PCR products. Also, amplification cycles might decrease the diversity of the final PCR product, owing to the loss of sequences that are amplified less efficiently; the consequence may be an erroneous assessment of the true cellular diversity.

Another method used to assess the T cell repertoire diversity is the frequency analysis of a given TCR clone based on techniques of limiting dilution [124]. This method is quite laborious and based on the not yet proven hypothesis that the frequency of a certain clonotype is representative of the frequency of all clones.

Lastly, a strategy frequently used in clinical research to access diversity is the analysis of lymphocyte populations by flow cytometry [125, 126]. This method is even less sensitive to detect alterations than the PCR-based methods, since the antibodies employed detect only the "constant" antigenic determinants shared by many lymphocyte receptor clones. Diversity evaluated by flow cytometry is, at best, inferred from the result.

In conclusion, several are the methods available to assess repertoire diversity but a direct, accurate, quantitative and easily used method is essential to examine and better understand the complexity of the immune system, both in physiologic and pathological situations, as the work presented in this dissertation demonstrates.

Chapter II

Aims of the study

The aims of this work and the hypothesis underneath these objectives were:

1) To develop a quantitative method to directly assess the T cell repertoire diversity.

    We hypothesize that it is possible to direct assess a cellular repertoire diversity by hybridization of all lymphocyte receptor-specific RNAs in a given sample to oligonucleotides on a gene chip as the number of sites undergoing hybridization out of the >400,000 available sites on a gene chip corresponds to the level of diversity.

2) To determine the role of polyclonal and monoclonal B cells and immunoglobulins upon T cell development, mostly the generation of the T cell repertoire diversity, using a mouse model where B cells have different levels of BCR diversity.

    We hypothesize that polyclonal B cells and polyclonal immunoglobulin have a fundamental role of promoting T cell development. This is because the diverse peptides from the immunoglobulin molecule can easily be presented to the T cells precursors in the thymus throughout the process of T cell development.

3) To examine the importance of the T cell repertoire diversity to peripheral T cells function through *in vitro* and *in vivo* testing.

   We hypothesize that the function of T cells may be improved if the diversity of the T cell repertoire is increased, since a larger range of distinct antigens would be detected by a more diverse T cell compartment as compared to a less diverse T cell repertoire.

4) To study the impact of lymphocyte and polyclonal immunoglobulins recovery in the survival of patients with certain hematological neoplasms, namely mantle cell lymphoma and multiple myeloma patients subjected to autologous stem cell transplantation.

   We hypothesize that the kinetics of the recovery of lymphocyte number during immune reconstitution may predict the robustness of the immune system translating in longer survival of the patients under these conditions; autologous hematopoietic stem cell transplantation provides a useful model to evaluate a regenerating immune system.

   Also, when a B cell neoplasm secreting monoclonal immunoglobulins is present (for example multiple myeloma), the recovery of a regular production of polyclonal immunoglobulin will indicate a better immunity and a better response to treatment.

5) To analyse the impact of the lymphocytes counts and sub-populations before treatment with rituximab in the progression free survival of patients with non-Hodgkin lymphoma receiving this antibody.

We hypothesize that the presence of a strong immune system will allow an optimization of the effects of anti-neoplastic treatment with rituximab, as the mechanisms of action of this antibody depend on the activity of elements of the immune system. So, the improvement of patients' immunity before treatment with rituximab should be actively sought.

CHAPTER III

Direct measurement of lymphocyte receptor diversity

Direct Measurement of Lymphocyte Receptor Diversity

Brenda M. Ogle[1,2], Marilia Cascalho,[1,3,4] Cristina M. Joao[1], William Taylor[5], Lori J. West[6] and Jeffrey L. Platt[1,3,4,7]

[1]Transplantation Biology Program, [2]Departments of Physiology, [3]Immunology and [4]Pediatrics, the [5]Cancer Center and [7]Surgery
Mayo Clinic, Rochester, Minnesota
[6]The Hospital for Sick Children Research Institute and the University of Toronto, Toronto, ON, Canada

This work was supported by grants from the National Institutes of Health (HL46810 and HL52297).

Nucleic Acids Research 2003, Vol. 31, No22 e129

3.1. Abstract

The ability to mount an immune defense against infectious microorganisms and their products and against tumors is believed to be a direct function of lymphocyte diversity. Because the diversity of lymphocyte receptor genes is >1000-fold more diverse than the entire genome and varies between genetically identical individuals, measuring lymphocyte diversity has been a daunting challenge. We developed a novel technique for measuring lymphocyte diversity directly using gene chips. We reasoned and here demonstrate that the frequency of hybridization of nucleic acids coding for lymphocyte receptors to the oligonucleotides on a gene chip varies in direct proportion to diversity. We applied the technique to detect changes in lymphocyte diversity in mice with known B cell alterations and in persons with known T cell repertoire defects. This approach is the first to provide direct analysis of lymphocyte receptor diversity and should facilitate fundamental study of the adaptive immune system and clinical efforts to assess immunological diseases. In addition this approach could be more broadly applied for example, to measure diversity of viral quasispecies.

Keywords: lymphocyte antigen receptor gene rearrangement, T lymphocytes, B lymphocytes, gene chips, oligonucleotide array sequence analysis

3.2. Introduction

While the total number of lymphocytes in the blood can be directly measured, the diversity of the lymphocyte compartment, on which immunocompetence is based, cannot. In the absence of direct measures of lymphocyte diversity, various indirect means for estimating diversity have been used. For example, antibodies against variable (V)-region families have been used to characterize lymphocyte populations by flow cytometric analysis [125, 127]. Since this approach detects "constant" antigenic determinants shared by many lymphocyte receptor clones, diversity is at best inferred from the result. As another example, nucleic acids encoding lymphocyte receptors can be amplified by PCR using constant region (C) and V-family specific primers [122]. Like FACS analysis, this approach does not differentiate between individual clones of the same family and may fail to detect balanced narrowing (or expansion) of the repertoire.

Diversity can also be estimated by spectratyping (also called immunoscope) or by the heteroduplex method [117, 118, 128]. These methods employ electrophoretic separation of amplified lymphocyte receptor V family PCR products according to the junctional (J) sequences; diversity is inferred from the number and electrophoretic separation pattern of amplified and re-annealed V region PCR products. Spectratyping and heteroduplex

methodologies effectively detect clonal expansion within a V family; however, because several thousand V-J family combinations for lymphocyte receptors exist, all V-J combinations cannot be analyzed routinely. Since only a small fraction of V-J combinations are analyzed, the choice of which is random, the actual diversity of the TCR repertoire may not be quantified.

Still another means to assess lymphocyte diversity is based on the tenants of limiting dilution analysis and detects the frequency of a given TCR clone [124]. This method is quite laborious and is based on the assumption that the frequency of the selected clonotype is representative of the frequency of all clones.

While techniques in current use offer value, they also have limitations, the most vexing of which is the inability to directly measure diversity. The approach we describe addresses this limitation by directly probing the entire population of lymphocyte receptors. This is accomplished by hybridization of all lymphocyte receptor-specific RNAs in a given sample to oligonucleotides on a gene chip; the number of sites undergoing hybridization out of the >400,000 available sites on a gene chip corresponds to the level of diversity. This approach sidesteps analysis of specific receptor families or clones and the limitations associated therein.

3.3. Materials and Methods

3.3.1. Isolation of RNA. All mouse strains were raised and maintained with protocols approved by the institutional animal care and use committee of the Mayo Clinic, Rochester, MN. All human samples were obtained in accordance with the institutional review board of the Mayo Clinic, Rochester, MN. Spleens harvested from mice were placed in RPMI and pushed through a 70 μm cell strainer. Lymphocytes were isolated from the resulting suspension of splenocytes or from peripheral blood using Ficoll-paque™ (Amersham Biosciences, Piscatanaway, New Jersey) gradient. Total RNA was isolated from the lymphocytes using the Qiagen RNeasy kit™ (Qiagen, Inc., Valencia, California) per the manufacturer's instructions. Isolated RNA was resuspended at a concentration of 2 μg/μl.

3.3.2. Generation of lymphocyte receptor-specific cRNA. First strand cDNA was constructed first as follows. In an RNAse-free microcentrifuge tube, 10 μl of total RNA (20 μg) was mixed with 1 μl (100 pmol/μl) of either:

T7+Cβ

(5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGCGGGCTGCTCC TTGAGGGGCTGCG-3') for T cell receptor analysis or

T7+CJ$_{H4}$

(5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGGAGGAGACGG TGACTGAGGTTCCTTG-3') for B cell receptor analysis.

This mixture was incubated at 70°C for 10 minutes followed by a quick spin and chill on ice. To this reaction, 4 μl of 5X first strand cDNA buffer (Invitrogen, Inc., Carlsbad, California), 2 μl of 0.1 M DTT, and 1 μl of 10 mM dNTP mix were added and incubated at 37°C for 2 minutes. Next, 2 μl of SuperScript II Reverse Transcriptase™ (Invitrogen, Inc.) was added and the total mixture was further incubated at 37°C for 1 hour. Following incubation, the first strand product was placed on ice. For second strand synthesis, the following reagents were added to the first strand product: 91 μl of DEPC-treated water, 30 μl of 5X Second Strand Reaction Buffer (Invitrogen, Inc.), 3 μl of 10mM dNTPs, 1 μl of 10U/μl DNA ligase, 4 μl of 10U/μl DNA polymerase I, and 1 μl of 2U/μl RNase H. The reaction was incubated at 16°C for 2 hours in a cooling water bath. Following incubation, 2 μl of 10U T4 DNA polymerase was added and the entire mixture was incubated for an additional 5 minutes at 16°C. Finally, 10 μl of 0.5 M EDTA was added to the mixture. The completed double-stranded cDNA was purified using phase lock gel followed by phenol chloroform extraction. The double-stranded cDNA product was then biotinylated with Enzo, BioArray High Yield RNA Transcript Labeling Kit™ per the manufacturer's instructions. The IVT product (cRNA) was purified using RNeasy spin columns (Qiagen, Inc.) per the manufacturer's

instructions. The purified product was quantified using spectrophotometric analysis applying the convention that 1 OD at 260 nm equals 40 μg/ml of RNA. cRNA was resuspended at a concentration of 1 μg/μl. cRNA was then fragmented to 50-200 bp sizes by combining with 5 μl of 5X fragmentation buffer (Invitrogen, Inc.) in 15 μl of water. The mixture was incubated at 94°C for 35 minutes and put on ice following incubation.

3.3.3. Application of cRNA to the gene chip. Equal amounts of cRNA from different samples were hybridized to U95B gene chips (Affymetrix, Inc., Santa Clara, California). While the ideal gene chip might be constructed using random oligonucleotides, we reasoned that chips containing known but unselected expressed sequence tags from human genes would share less homology with mouse lymphocyte receptor RNA and could be used instead. And in fact, duplicate experiments performed on U95C chips yielded comparable results, suggesting that a random oligonucleotide chip may not add benefit.

3.3.4. Data Analysis. For each gene chip experiment, we obtained raw data corresponding to oligo location and hybridization intensity. Data were arranged in order of ascending hybridization intensity. The number of oligo locations with intensity above background (i.e., number of hits) was

summed. First, the standard curve was generated (from hybridization of samples with known numbers of different oligos). Next, test samples were assessed and based on the number of hits, the diversity was extrapolated from the standard curve.

3.3.5. ELISA for detection of anti-KLH antibodies. Mice were immunized by intraperitoneal injection with 25 μg of KLH (Keyhole Limpet Hemocyanin, Sigma, St. Louis, MO) in incomplete Freund's adjuvant. A boost of 10 μg of KLH was administered 20 days later. After an additional two weeks the mice were sacrificed and serum and splenocytes were isolated. Purified KLH (3 μg/ml in phosphate buffered saline (PBS); 50 μl/well) was added to wells of ninety-six well flat bottom microtiter plates (Nunc-Immuno 96 Micro well – Maxisorp™, Nalge Nunc International, Rochester, NY). ELISA was developed as described [129]. Plates were read using a microplate reader (Power Wave X™, Bio Tek Instruments, Winooski, VT) and analyzed using KC4 – Kineticalc software. Samples were analyzed in triplicate in three independent experiments.

3.3.6. Statistical Analysis. Slope and y intercept of the standard curves were compared between experiments using a single-factor analysis of variance for a random-effects model. Differences were considered significant

at a value of $p < 0.05$. Immune responsiveness was compared between control (C57) and various mutant mice (QM, $J_H^{-/-}$) using unpaired Student's *t* test data. Differences were considered significant at a value of $p < 0.05$. All gene chip experiments were performed twice; representative data are shown.

3.4. Results

As a first test of the concept, we asked whether the diversity of random oligonucleotides is predicted by the number of sites hybridized on a gene chip. Since the variable sequences of the complementarity determining region of lymphocyte receptors are relatively random, the sequences should be represented by random oligonucleotides. We designed a random oligonucleotide and then inserted random point assignments at specific locations along the oligonucleotide. For example, to generate a sample with ~10^6 different oligonucleotides, we synthesized the oligonucleotide with 10 sites of random base assignment (4^{10} = 1,048,576). We synthesized samples with 1, 10^3, 10^6, and 10^9 different oligonucleotides per sample. The oligonucleotides were biotinylated, and then 10 μg of each was hybridized to separate gene chips under similar stringency conditions to those for conventional applications. As anticipated, the number of hybridized sites increased with increasing diversity of oligonucleotides (Fig. 1A). Due to the exponential nature of the relationship, the natural logarithm of both variables was taken and plotted to yield a linear relationship (Fig. 1B, 1C).

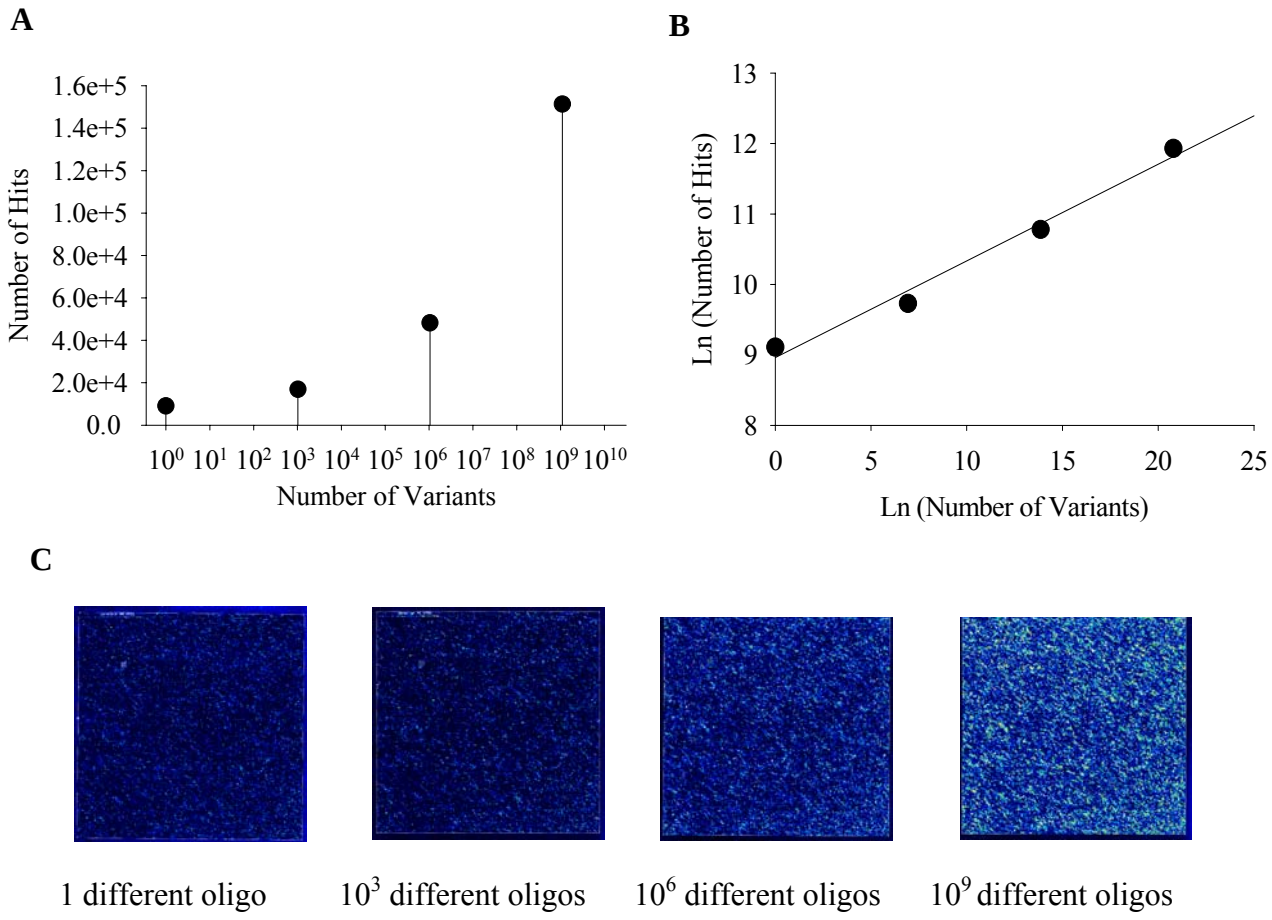


Figure 1. **Establishing the relationship between number of gene chip hybridization sites and sample diversity.** Samples consisting of 1, 10^3, 10^6, and 10^9 different oligos were biotinylated and hybridized to gene chips. Hybridization intensity data were arranged in ascending order. The number of probe locations with intensity above background (i.e., number of hits) was summed and compared to the number of different oligos initially applied to the gene chip (i.e., number of variants) (a) Relationship between number of hits and number of variants. The number of hits increases with the number of variants, indicating that the human gene chip can be used to detect random oligos. (b) Linear relationship between number of hits and number of

variants. The natural log of both axes yielded a linear relationship between hits and variants. (c) Visual hybridization of random oligos to gene chips. Scans of the gene chips afford rapid inspection of the "hit" profile.

This relationship represents a standard curve that can be used for assessment of diversity in physiologic samples. To our knowledge this approach is the only means to generate such a curve in the absence of physiologic samples of known diversity. To ascertain the reproducibility of this relationship (i.e., the standard curve), we repeated the above experiment six times (Fig. 2). The trend (i.e., slope of the standard curve) was highly reproducible ($p > 0.1$), however overall hybridization intensity (i.e., y intercept of the standard curve) varied between experiments ($p < 0.05$; Fig. 2). This variability requires use of a standard curve for each experiment conducted.

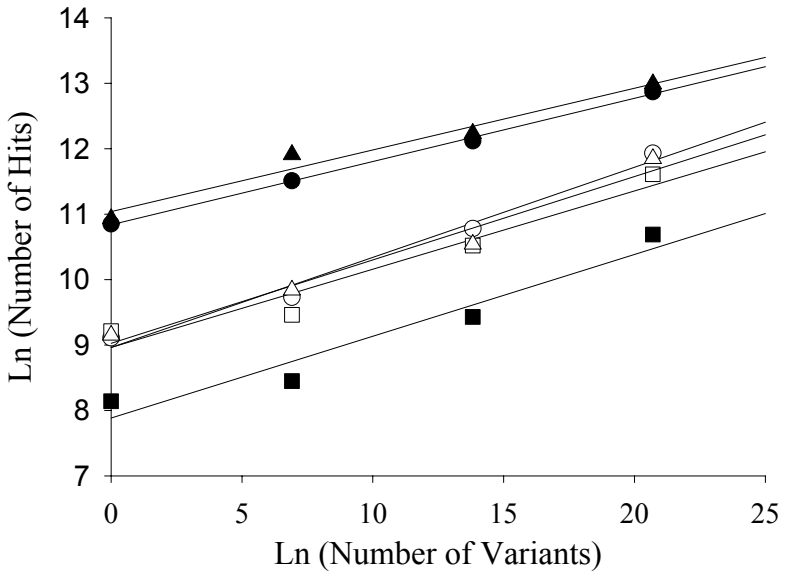


Figure 2. Reproducibility of method for analysis of receptor diversity. Samples obtained as described in Figure 1 were studied in six separate experiments (black circle,-●-)(white circle, -○-)(black square, -■-)(white square, -□-)(black triangle, -▲-)(white triangle, -Δ-) to test reproducibility.

The slopes of the standard curves were the same statistically; however, the y intercept varied from experiment to experiment.

To test whether variations in lymphocyte diversity could be measured directly, we the method to the study of B cells in mice. We used murine B cells for this purpose because diversity of these cells can be measured, at least in principle, through analysis of Ig gene expression and because we have available mutant mice with defined variations in B cell antigen receptor repertoire.

We compared diversity of B cell antigen receptors in wild type (C57Bl/6) mice with the diversity of B cell receptors in quasi-monoclonal (QM) and $J_H^{-/-}$ mice. The QM mice were generated by gene-targeted replacement of the endogenous J_H elements with a VDJ rearranged region from a (4-hydroxy-3-nitrophenyl) acetyl (NP)-specific hybridoma [130]. The kappa light chains in these mice are non-functional and, therefore, the knock-in heavy chain can only pair with endogenously rearranged lambda chains. All B cells in QM mice start out with the same heavy chain (QM B cells); however, secondary rearrangements and hypermutation change the specificity of B cell receptors (non-QM B cells) [130]. Thus, 80% of the peripheral B cells in the QM mice express NP-specific antibodies that contain the same single heavy chain, and the remaining 20% express antibodies expressing diverse heavy chains and diverse antigen specificities. $J_H^{-/-}$ mice have a targeted deletion of the J_H and

of the J_{kappa} gene segments and, therefore, cannot assemble Ig heavy or kappa light chains [131]. These animals are B cell deficient although they do have surface Ig-negative precursor B cells ($B220^+/CD43^+$ pro-B cells) that assemble lambda light chain genes at a low level.

To test changes in lymphocyte diversity, we compared gene chip hybridization of B cell heavy chain-specific cRNA from splenocytes of wild type, QM and $J_H^{-/-}$ mice. The heavy chain specificity of the cRNA was gained via generation of first strand cDNA from isolated RNA using a J_{H4}-specific custom primer. A primer to the J_{H4} region of the heavy chain was used to detect diversity in one region of the heavy chain. While the results do not represent diversity of the entire heavy chain population they might provide a baseline from which a change could be detected. Following second strand DNA synthesis, the double-stranded product was biotinylated via *in vitro* transcription (IVT). The IVT product (cRNA) was purified and 10 μg of each sample and 10 μg of each standard were hybridized to individual gene chips. The hybridization intensities obtained from the $J_H^{-/-}$ mice (which lack B cell receptors) were used to set the background threshold, above which hybridization sites (hits) were counted. Sample diversity was extrapolated from the standard curve.

As the results shown indicate, wild type mice expressed more than 10^5 (2.8×10^5) different B cell J_{H4}-positive heavy chain clones and QM mice expressed 3.9×10^2 different J_{H4}-positive heavy chain clones (Fig. 3A). Thus, as

expected, QM J_{H4}-positive heavy chain diversity was much less than wild type diversity, though well above background levels.

We then tested the ability of this system to detect changes in diversity following antigenic challenge with keyhole limpet hemocyanin (KLH).

Following immunization with proteins such as KLH, heavy chain diversity is thought to decrease due to oligoclonal expansion of high affinity clones. In contrast, immunization of QM mice with KLH (an antigen that does not bind with the QM antibody) causes diverse non-QM B cells to expand at the expense of the predominant QM B cells, thereby increasing heavy chain diversity [132]. Consistent with these theoretical concepts, J_{H4}-positive heavy chain diversity in wild type mice decreased by greater than one order of magnitude, from 2.8×10^5 to 4.0×10^4, and diversity in QM mice increased (7.5×10^3) following immunization with KLH

(Fig. 3A). Immune responsiveness was confirmed by quantitation of anti-KLH Ig in the serum (Fig. 3B).

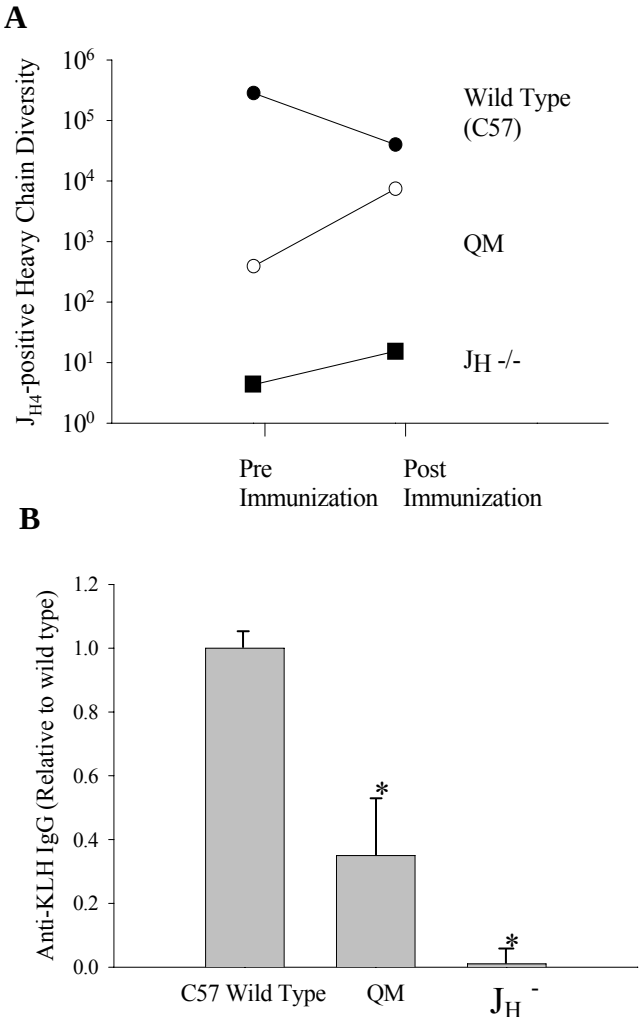


Figure 3. Analysis of B cell diversity using the gene chip method. Splenocytes were harvested from 3-4 week old J_H-/-, QM and WT mice and mononuclear cells were isolated on Ficoll-paque gradients. Total RNA was isolated from the lymphocytes and first strand cDNA was generated using a primer designed to bind the constant region of the mouse heavy chain J_{H4} region plus the T7 polymerase promoter. The custom primer promoted amplification of J_{H4}-heavy chain-specific RNA only. Equal amounts of the *in*

vitro transcription product (cRNA) from each mouse and standards were hybridized to gene chips and then the chips were stained and analyzed as described in the methods. (a) B cell heavy chain diversity in mutant mice before and after immunization with KLH. Pre-immunization, WT diversity (black circle, -●-) was more than two-fold higher than QM (white circle, -○-) diversity. Post-immunization, WT diversity (black circle, -●-) decreased (4.0 x 10^4 different B cell heavy chain clones) while QM (white circle, -○-) diversity increased (7.5 x 10^3). Background hybridization was established using $J_H^{-/-}$ RNA (black square, -■-). (b) Immune responsiveness to KLH. An ELISA was used to detect levels of anti-KLH antibodies in the serum following immunization. The QM anti-KLH antibody titer was approximately 40% of the wild type following immunization: * $p < 0.05$.

We next asked whether this approach could distinguish medically relevant conditions in humans via assessment of T cell diversity. Peripheral blood lymphocytes were isolated from two normal individuals (35 and 55 years of age) and from two individuals who had cardiac transplants in infancy (3 and 5 years of age; transplantation was conducted at < 1 year of age). Those who receive cardiac transplants in infancy undergo removal of the thymus as part of the surgical procedure and depletion of T cells with anti-T cell

antibodies [133]. These patients would be expected to have a notably contracted TCR repertoire because they lack a source of new naïve T cells.

RNA was isolated from these lymphocytes and from Jurkat cells and first strand cDNA was generated using a primer designed to bind the constant region of the TCR beta chain. We could have designed a primer to bind the constant region of the TCR alpha chain, or combined the alpha chain and beta chain primers to isolate all TCR RNA. However, the majority of lymphocyte diversity is conferred by the beta chain [22] and so in an effort to maintain the simplicity of the system, here we show beta chain diversity only. Jurkat cells express only one TCR (Vβ1.2Vβ8.1), and so the hybridization intensities of this sample were used to establish the background threshold.

Normal human T cell receptor beta chain diversity was 4.4×10^6 and 5.1×10^6 (Normal; Fig. 4). These values are consistent with estimates of beta chain diversity deduced by other means [124, 134] and taken with estimates of β-chain diversity and pairing [22] would place overall T cell diversity at minimally 1.1×10^8 (4.4×10^6 * 25 alpha families). And as expected, the T cell receptor beta chain diversity of the thymectomized/T cell depleted subjects (2.2×10^3 and 1.8×10^2; Thymectomized/T cell depleted, Fig. 4) was more than two orders of magnitude lower than that of normal individuals. To determine whether the assay could detect smaller changes in T cell diversity, we tested an individual with inflammatory bowel disease (25

years of age), which in experimental systems has been associated with decreased T cell diversity [135]. We found a decrease in TCRβ diversity of more than one order of magnitude in this subject (2.6 x 10^5; IBD, Fig. 4). Our results thus suggest that human inflammatory bowel disease may be linked with decreased lymphocyte diversity.

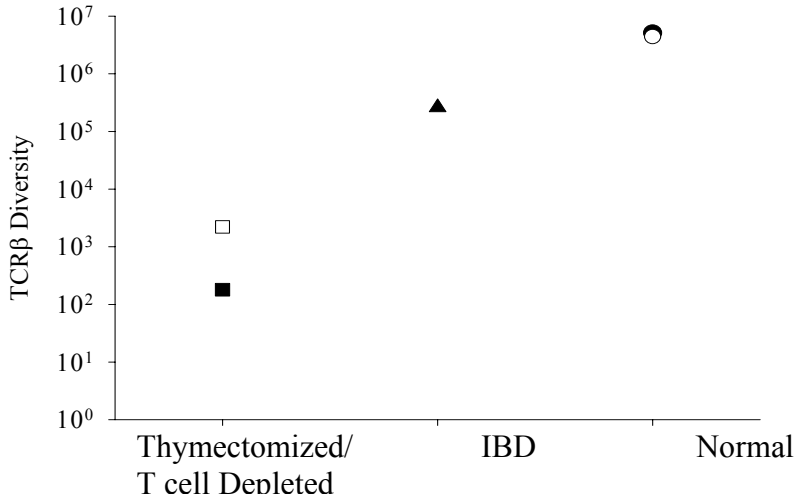


Figure 4. Analysis of human T cell diversity using gene chips. Total RNA was isolated from human peripheral blood lymphocytes or Jurkat cells and first strand cDNA was generated using a primer designed to bind the constant region of the TCRβ chain.

Equal amounts of the *in vitro* transcription product (cRNA) from each sample and standards were hybridized to gene chips and then the chips were stained and analyzed as described in the methods. Two normal individuals [(black circle, -●-), (white circle, -○-)], two thymectomized/T cell depleted individuals [(black square, -■-), (white square, -□-)] and one individual with IBD (black triangle, -▲-) were analyzed. The TCRβ chain diversity of the normal individuals was 4.4×10^6 and 5.1×10^6 respectively (similar results were found even after diluting the normal sample with Jurkat cells at various ratios up to 20:80), the thymectomized individuals 2.2×10^3 and 1.8×10^2 respectively and the individual with IBD 2.6×10^5. Background hybridization was established using Jurkat cell hybridization.

3.5. Discussion

Here we report the direct measurement of lymphocyte diversity and illustrate potential applications. The system we devised can estimate diversity of the entire lymphocyte repertoire (i.e., all gene segment combinations) at once and avoids the complications of indirect estimates of lymphocyte diversity. With only minor adjustments, this approach is equally capable of measuring B cell and T cell diversity and can be adjusted via primer design to include or exclude lymphocyte receptor subsets. The system is sufficiently simple and effective to allow widespread application.

There is much interest in the relationship between lymphocyte diversity and immunocompetence. Loss of diversity has been implicated in various disease states [124, 136, 137], and so changes in diversity might be used to track the progression or remission of disease. For example, this approach might be used to monitor immune reconstitution following bone marrow transplantation or intensive retroviral therapy. In these settings, a small number of clones might expand by homeostatic proliferation to yield normal lymphocyte numbers, but diversity might be altered [138]. The technique reported here might also be used to track expanded T cell clones or clusters of clones over time based on gene chip hybridization pattern. Certainly, the numbers of individual T cell clones in normal blood are too few to allow clone tracking by any means; however, the expanded T cell clones seen in

response to infection, transplantation or homeostatic proliferation might in principle be tracked over time with this method. Preliminary experiments done in our laboratory support this concept (data not shown).

While we have focused on the measurement of lymphocyte receptor diversity, the technique here discussed might also be applied more broadly to the daunting task of quantifying biologic diversity of, for example, viral quasispecies. Because persistence of some viral infections such as hepatitis C is positively related to the diversity of the virus [139], quantifying quasispecies diversity may be critical to guide therapeutic choices and prognostic assessments. Diversity of hepatitis C virus quasispecies may be accomplished by generating viral envelop gene-specific cRNA followed by hybridization to gene chips and analysis similar to the one described above for the lymphocyte receptor genes.

The primary challenge of using gene chips to measure lymphocyte diversity is determining the background correction for analysis of extremely narrow repertoires. We are exploring the usefulness of intensity data from non-lymphoid cells for this purpose as well as enhancing our panel of standards to include all single order variations in diversity (i.e., 10^1, 10^2, 10^4, 10^5, etc.). An additional caveat warrants mention. It is difficult to predict whether the number of hits generated by a diverse oligonucleotide mixture is equivalent to the number of hits generated by the same diversity of lymphocyte

receptor cRNA. We controlled for the potential difference in hybridization by including a sample of known diversity (i.e., Jurkat with 1 TCR and MBT with 1 BCR) in the generation of the standard curve. Thus the number deduced by this approach, while likely not the actual number of different receptors in a population, is an approximation of the actual number of receptors in a population. The number therefore serves both as an estimate of the receptor diversity within a population and a basis for comparison with other populations.

Overall, the advent of high-throughput hybridization technology has made it possible to directly assess lymphocyte receptor diversity and thus perhaps immune fitness. This approach should facilitate fundamental study of the physiology of the adaptive immune system and clinical efforts to asses and follow immunological diseases.

CHAPTER IV

B cell-dependent TCR diversification

B cell-dependent TCR diversification

Cristina João[*], Brenda M. Ogle[*,†], Carlota Gay-Rabinstein[*], Jeffrey L. Platt[*,‡,§,¶], Marilia Cascalho[*,‡,§,¶]

Transplantation Biology Program[*], and the Departments of Physiology[†], Immunology[‡], Surgery[§] and Pediatrics[¶]
Mayo Clinic, 200 First Street SW, Medical Sciences Building 2-63,
Rochester, Minnesota 55905

This work was supported by the grants from the National Institutes of Health (AI48602, AI41570 and HL46810) and by a grant (SFRH/BD/6797/2001) from the Fundação para a Ciência e Tecnologia – Portugal.

Journal of Immunology 2004,172:4709-4716

4.1. Abstract

T cell diversity was once thought to depend on the interaction of T cell precursors with thymic epithelial cells. Recent evidence suggests, however, that diversity might arise through the interaction of developing T cells with other cells, the identity of which is not known. In this study we show that T cell diversity is driven by B cells and Ig. The TCR Vβ diversity of thymocytes in mice that lack B cells and Ig is reduced to 6×10^2 from wild-type values of 1.1×10^8; in mice with oligoclonal B cells, the TCR Vβ diversity of thymocytes is 0.01% that in wild-type mice. Adoptive transfer of diverse B cells or administration of polyclonal Ig increases thymocyte diversity in mice that lack B cells 8- and 7-fold, respectively, whereas adoptive transfer of monoclonal B cells or monoclonal Ig does not. These findings reveal a heretofore unrecognized and vital function of B cells and Ig for generation of T cell diversity and suggest a potential approach to immune reconstitution.

Keywords: T cell repertoire, antigen receptor diversity, B cell deficiency, immunoglobulin, thymus.

4.2. Introduction

The generation of TCR diversity is initiated by recombination of the V, D, and J gene segments and originates the variable region of the TCR genes in T cell precursors in the thymus [21]. Although V(D)J recombination generates billions of different TCRs, only a small fraction of these (5%) is expressed by the mature thymocytes [140]. Thymocytes that fail to produce TCR or that produce TCR that fails to recognize MHC bearing self peptide die by neglect. Thymocytes bearing self-reactive TCR are eliminated (negative selection), leaving a small fraction of thymocytes surviving (positive selection) [140]. Thus, positive and negative selection give rise to a primary T cell repertoire that recognizes self-MHC (restriction) with moderate avidity that is not self-reactive and, in turn, establishes the diversity of naive T cells. Therefore, assuming an equal contribution of V(D)J recombination, the diversity of newly made thymocytes reflects the efficiency of selection. T cell diversity has been estimated to be 10^8 different T cells in humans and 10^6 in mice [22, 24].

Positive selection and T cell restriction have been thought to result from the interaction of developing T cells with thymic epithelial cells [141]. This conclusion was deduced from experiments in which lethally irradiated recipient mice of different MHC haplotypes were reconstituted with bone marrow cells obtained from H-2b TCR transgenic mice. In these chimeras, T cells were positively selected only when the thymic MHC was of the H-2b

haplotype, indicating that H-2b expression by bone marrow-derived cells alone was not sufficient to promote positive selection [15]. The conclusion that thymic epithelial cells mediate positive selection was also indicated by the work of Benoist and Mathis [142], who showed that the expression of MHC class II on cortical thymic epithelium was sufficient to achieve positive selection of thymocytes, whereas the expression of the same MHC antigens on hematopoietic-derived cells was not.

While thymic epithelium might be sufficient to mediate positive selection, some wondered whether cells other than thymic epithelial cells could also participate in positive selection. Pawlowski et al. [143] and Hugo et al. [144] showed in separate experiments that MHC class I- or MHC class II-bearing fibroblasts injected into the thymus of MHC class I-deficient or MHC class II-deficient mice were able to mediate positive selection. Bix and Raulet [145] showed that bone marrow-derived cells in MHC class I-deficient bone marrow chimeras promoted positive selection of CD8+ thymocytes. In contrast, MHC class II-positive bone marrow-derived cells did not rescue CD4+ T cells in MHC class II-deficient mice [146]. If some experiments establish that positive selection can, in some cases, be mediated by nonthymic epithelial cells, the question of how physiologic these interactions may be is not resolved. Zinkernagel and collaborators generated tetraparental aggregation chimeras in which thymic epithelial cells expressed one MHC, and hematopoietic-derived cells expressed another [147]. In these chimeras, T cells were restricted to the MHC expressed on the thymic epithelial cells as

expected, but also to the MHC expressed by the hematopoietic cells. These results indicated that nonthymic epithelial cells as well as thymic epithelial cells promote positive selection of thymocytes [147]. Which hematopoietic-derived cells were responsible for the positive selection of thymocytes was not determined.

Besides the question of which cells mediate positive selection of thymocytes is the question of which cells provide the source of peptides. One speculation has been that the peptides originate in the thymic epithelium [148]; however, the repertoire of peptides available from this source may not suffice. Selection of a diverse T cell repertoire requires diverse peptides presented in the context of self-MHC. Thus, mice that express MHC associated with a single peptide have a markedly constrained T cell repertoire [149-151].

As B cells are normal constituents of the thymus [152] and may present peptides derived from the Ig V regions [153] or peptides derived from antigens expressed endogenously [154], we hypothesized that B cells would potentially serve as a source of peptide diversity in the thymus. In fact, B cells are one of the major cell types expressing MHC class II and are capable of presenting antigens to T cells [100]. We tested this hypothesis by comparing the diversity of the TCR repertoire in mice with varying B cell numbers and varying B cell diversity and by testing whether transfer of B cells or immunoglobulin could rescue TCR diversity. Our studies demonstrate

that B cells and immunoglobulin generate a significant fraction of T cell diversity.

4.3. Materials and Methods

4.3.1. Mice strains. JH-/-, μMT, monoclonal B-T, and quasi-monoclonal (QM) mice were previously described [19, 129, 131, 155]. The B cell-deficient strains consisted of JH-/- mice, obtained by gene-targeted deletion of the JH segments [131], and μMT mice, obtained by gene-targeted disruption of the μ Ig H chain membrane exons [155]. C57BL/6 and μMT mice were purchased from The Jackson Laboratory (Bar Harbor, ME). JH-/-, monoclonal B-T, and QM mice were bred and all mice were housed in a specific pathogen-free facility at Mayo Clinic (Rochester, MN). All mice were between 6 and 16 wk of age and were age-matched. All animal experiments were conducted in accordance with protocols approved by Mayo Clinic Institutional Animal Care and Use Committee.

4.3.2. Adoptive transfer of B cells. Bone marrow cells were harvested from C57BL/6 mice or monoclonal B-T mice, and lymphocytes were isolated by Ficoll-Paque gradient (Amersham Pharmacia Biotech, Piscataway, NJ). Polyclonal B cells (1 x 107) purified with a Miltenyi Biotec isolation kit (Auburn, CA) and monoclonal B cells (6 x 106) purified with a high speed sorter FACSVantage SE (BD Biosciences, Mountain View, CA) were injected i.p. in newborn mice (20 μl). The polyclonal B cells contained, on the

average, 1% CD3+CD4+ and 1.5% CD3+CD8+ cells, and the monoclonal B cells contained 0% CD3+CD4+ and 0.07% CD3+CD8+ cells.

4.3.3. Immunoglobulin injections. JH-/- mice were injected i.p. weekly with 250 μg of mouse polyclonal IgG (Serotec, Oxford, U.K.) or monoclonal anti-keyhole limpet hemocyanin IgG2b (C48-4; BD Biosciences) from birth. Serum levels of total Ig were tested 4 wk or more after the first injection.

4.3.4. Flow cytometry analysis. Thymocytes were obtained by mincing thymi through a 0.70-μm pore size mesh, followed by RBC hemolysis in a standard NH4Cl lysis buffer. Bone marrow cells were prepared by flushing femurs with cell suspension buffer, followed by RBC lysis, as previously described [129]. Total thymocyte numbers were counted with a Coulter counter (Hialeah, FL). Cells were stained with one, two, or three of the following monoclonal antibodies (all the antibodies were from BD PharMingen, San Diego, CA) as previously described [129]. FITC-conjugated rat anti-mouse CD4 (GK 1.5), rat anti-mouse CD43 (Ly-48, leukosialin), and mouse anti-5-bromo-2'-deoxyuridine (anti-BrdU) antibodies; PE-conjugated rat anti-mouse CD8 (53-6.7), rat anti-mouse CD19 (1D3), and rat anti-mouse IgMb (Igh-6b; AF6-78); and biotin-conjugated rat anti-mouse B220 (16A), rat anti-mouse CD62L (LECAM-1, Ly22), and rat anti-mouse CD3

(145-2C11). Lymphocytes were gated on the light scatter plot by back-gating onto CD4+CD3+ and CD8+CD3+ cells; the numbers of the thymocyte subpopulations were determined by multiplying the percentage, as defined by gating on the FACS plot, by their total number.

4.3.5. DNA analysis. Thymocytes (106/ml) were washed with ice-cold PBS and fixed in 70% ethanol at -20°C for at least 2 h. After fixation, cells were washed twice with PBS and incubated in 50 µl of DNA extraction buffer (0.2 M phosphate citrate buffer, pH 7.8) at 37°C for 30 min in a shaker. After DNA extraction, the cells were stained with propidium iodide in a solution containing 10 ml of 0.1% (v/v) Triton X-100 in PBS, 200 µl of 1 mg/ml propidium iodide (Molecular Probes, Eugene, OR), and 2 mg of DNase-free RNase A (Sigma-Aldrich, St. Louis, MO), for 30 min at room temperature. Detection of propidium iodide fluorescence was read at red wavelength in a FACScan flow cytometer (BD Biosciences) and was analyzed with ModFit LT software (Verity Software House, Topsham, ME).

4.3.6. Immunohistological analysis. Thymi removed from 6- to 8-wk-old mice were oriented, covered with OCT (Sakura, Torrance, CA), snap-frozen by pre-cooled isopentane, and stored at -85°C. Four-micron-thick frozen sections were mounted on positively charged microscope slides (SuperFrost

Plus; Fisher Scientific, Pittsburgh, PA) and stored at -85°C. Before processing, sections were air-dried at room temperature, fixed 10 min in 4°C acetone, air-dried for an additional 10 min, then post-fixed for 2 min in 100 mM Tris-buffered 1% paraformaldehyde containing 1 mM EDTA, pH 7.2, and rinsed with PBS (pH 7.2). Before staining, the specimens were incubated in 0.3% hydrogen peroxide in 0.1% sodium azide (aq) solution to quench the presence of endogenous peroxidase. Specimens were incubated with rat monoclonal antibodies to murine CD19 (clone 1D3; BD PharMingen) and to CD45R/B220 (clone RA3-6B2; BD PharMingen), rinsed with PBS, then detected by mouse serum pre-absorbed, affinity-purified, biotinylated goat IgG anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), followed by PBS rinses and the tertiary application of horseradish-conjugated streptavidin (DAKO, Carpinteria, CA). Slides were developed by incubation with a peroxidase substrate NovaRED (Vector Laboratories, Burlingame, CA), which resulted in an insoluble reddish-brown precipitate, followed by counterstaining with a progressive alum-hematoxylin, dehydrated in graded ethanols, cleared in xylene changes, and coverslipped with Cytoseal-Xyl (Stephens Scientific, Kalamazoo, MI). Digital images were obtained using a brightfield microscope equipped with a CCD digital camera (SPOT II; Diagnostic Instruments, Sterling Heights, MI).

Apoptotic cells were detected in cryostat sections of thymi by in situ TUNEL, performed according to the manufacturer's instructions (ApopTag Plus Peroxidase Kit; Serologicals, Norcross, GA).

4.3.7. Determination of TCRVβ diversity

Isolation of RNA. Thymi harvested from mice were placed in RPMI 1640 and pushed through a 70-μm pore size cell strainer. Lymphocytes were isolated by Ficoll-Paque (Amersham Pharmacia Biotech) gradient. Total RNA was obtained with RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Generation of diversity standards. Diversity standards were prepared by creating oligonucleotide mixtures of known diversity. For example, to generate a standard diversity of 106, 18-mer oligonucleotides were synthesized with 10 sites of random assignment, generating 410 = 1,040,526 different oligomeres. Similarly, we created oligomer mixtures with 1, 103, and 109 variants. Oligonucleotides were biotin-labeled and hybridized to the gene chips as explained below.

Generation of lymphocyte receptor-specific cRNA. First-strand cDNA was obtained by RT with a mouse TCR C reverse primer, T7+C (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGCTTGGGTGGAGTCACA TTTCTC-3'). Second-strand synthesis and preparation of biotin-labeled cRNA were conducted according to standard protocols (Affymetrix, Santa Clara, CA).

Application of cRNA to the gene chip. Equal amounts of cRNA from different samples and diversity standards were hybridized to U95B gene chips (Affymetrix). Gene chips were processed at the Microarray Core Facility (Mayo Clinic).

Data analysis. For each gene chip experiment, we obtained raw data corresponding to oligo location and hybridization intensity. Data were arranged in order of ascending hybridization intensity. The number of oligo locations with intensity above background (i.e., number of hits) was summed. The standard curve was generated from hybridization of samples with known numbers of different oligomeres. The standard oligonucleotide mixtures were 18-mer oligomeres synthesized to obtain mixtures containing 1, 10^3, 10^6, and 10^9 different oligonucleotides. The diversity of cRNA obtained from monoclonal T cells was used to establish the background, and the diversity of the test samples was extrapolated directly from the standard curve. We controlled for the TCR specificity of the C reverse primer by determining the diversity of cRNA obtained from purified polyclonal B cells with the C reverse primer, which was found to be three per 10 μg of RNA and indistinguishable from background.

4.3.8. Statistical analysis. Statistical analysis for group comparison of means of TCR Vβ diversity of thymocytes was performed using log transformation of the data, followed by one-way ANOVA. Groups of two comparisons were performed by unpaired, two-sided Student's t test. Comparisons of thymocyte numbers were performed using the Kruskal-Wallis test for global differences, followed by the Wilcoxon rank-sum test. A value of $p < 0.05$ was considered statistically significant.

4.4. Results

4.4.1. Thymocyte development is perturbed in mice that lack B cells and Ig

To test whether B cells and/or Ig might contribute to thymic selection, we compared the numbers of thymocytes and thymocyte subpopulations in mice that lack B cells and Ig (JH-/-) with the numbers in wild-type mice (C57BL/6). Because the thymus atrophies with age, the mice in each group were age-matched. Our results show that JH-/- mice had significantly fewer total thymocytes (6.5-fold) than C57BL/6 mice (Fig. 1). The smaller number of thymocytes in JH-/- mice was mainly due to a 3.9-fold decrease in the number of CD4+CD8+ thymocytes (Fig. 1), but also reflected a significant decrease in the numbers of CD4- CD8-, CD4+ CD8-, and CD4- CD8+ populations compared with wild type. These results suggest that B cells and/or a B cell product such as Ig might influence thymocyte development by various direct or indirect means.

To determine whether Ig might on its own contribute to T cell development, we examined thymocytes in the μMT mouse, which has a serum Ig concentration 4.5% that of the wild-type mouse, but very few B cells [156] [157] (Table I). We found that the number of thymocytes in μMT mice was increased significantly by 3.2-fold compared with that in JH-/- mice (Fig. 1). These results are consistent with a role for Ig in the development of T cells.

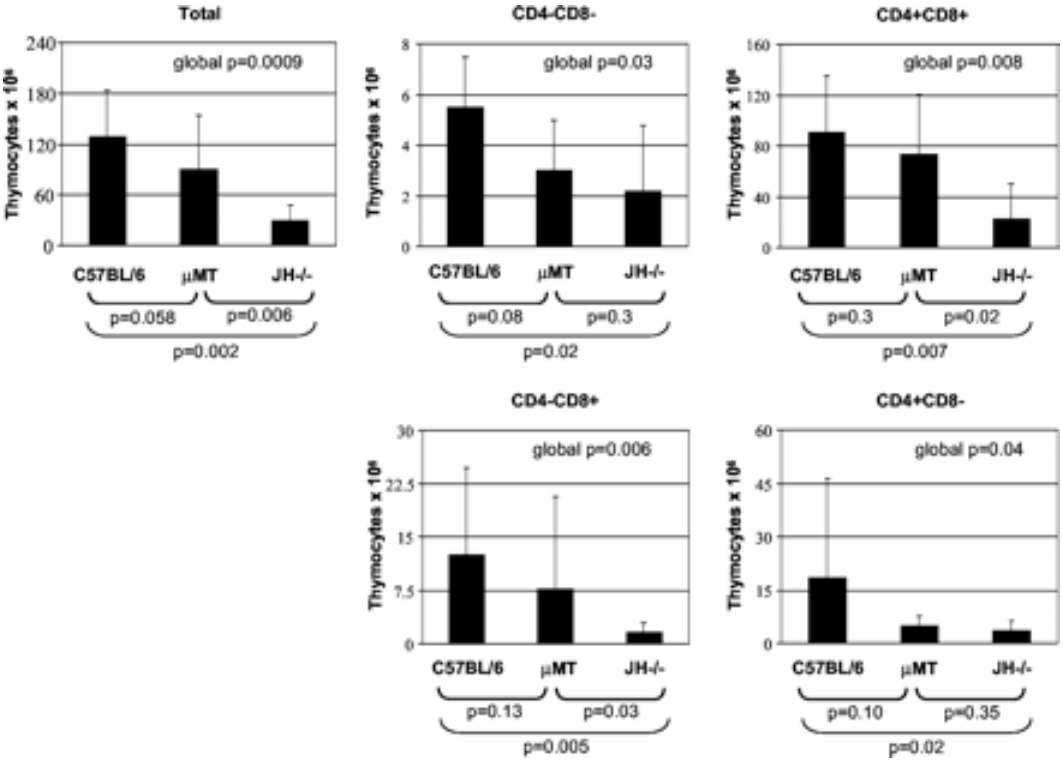


FIGURE 1. Thymocyte numbers in C57BL/6, µMT, and JH-/- mice. The number of thymocytes corresponding to each sub-population was calculated by multiplying the respective percentage of the total events as defined in the flow cytometric dot plot analysis by the total number of WBC obtained by counting on a Coulter counter. The number of thymocytes (average ± SD) was $1.3 \times 10^8 \pm 5.1 \times 10^7$ in C57BL/6, $8.9 \times 10^7 \pm 6.4 \times 10^7$ in µMT, and $3.1 \times 10^7 \pm 1.7 \times 10^7$ in JH-/- mice. The average number of CD4-CD8- thymocytes was $5.5 \times 10^6 \pm 2.1 \times 10^6$ in C57BL/6, $3 \times 10^6 \pm 2 \times 10^6$ in µMT, and $2.2 \times 10^6 \pm 2.7 \times 10^6$ in JH-/- mice; the average number of CD4+CD8+ thymocytes was $9 \times 10^7 \pm 4.4 \times 10^7$ in C57BL/6, $7.3 \times 10^7 \pm 4.7 \times 10^7$ in µMT, and $2.3 \times 10^7 \pm 2.7 \times 10^7$ in JH-/- mice; the average number of CD4-CD8+ thymocytes was $1.2 \times 10^7 \pm 1.2 \times 10^7$ in C57BL/6, $7.7 \times 10^6 \pm 1.3 \times 10^7$ in µMT, and $1.5 \times 10^6 \pm 1.4 \times 10^6$ in JH-/-mice; and the average number of CD4+CD8- thymocytes was $1.9 \times 10^7 \pm 2.8 \times 10^7$ in C57BL/6, $4.9 \times 10^6 \pm$

2.8 x 10^6 in μMT, and 3.5 x 10^6 ± 3 x 10^6 in JH-/- mice. Error bars represent the SD. Data were obtained from 10 C57BL/6, 8 μMT, and 10 JH-/- mice for the total number of thymocytes and from 8 C57BL/6, 8 μMT, and 7 JH-/- mice for thymocyte subpopulations. Mice were between 6 and 16 wk of age. Comparisons of the number of cells in the three strains of mice (indicated by global p) were performed by the Kruskal-Wallis test, and comparisons between two groups of mice (*p* values indicated below the diagrams) were made using the Wilcoxon rank-sum test.

Table 1. Mean concentration of serum Ig ± SD in C57BL/6, QM, μMT, JH-/- mice and in JH-/- mice reconstituted with B cells or after administration of IgG

Mice	**Serum Immunoglobulin (μg/ml)**
C57BL/6	163.5 ± 160.6
μMT	10.7 ± 24.7
QM	654.5 ± 248.8
JH-/-	0
JH-/- with Polyclonal B cells	0
JH-/- with Polyclonal IgG	832.2 ± 1380.1

4.4.2. Increased apoptosis in the thymic cortex of mice that lack B cells and Immunoglobulin

Next we asked whether the fewer thymocytes in JH-/- mice were the result of higher levels of cell death. Consistent with that concept, TUNEL, which detects DNA strand breaks in cells undergoing apoptosis, revealed increased apoptosis (at least 2.5-fold) in the thymic cortex of JH-/- mice compared with μMT or to C57BL/6 mice (Fig. 2). Increased cell death could be the consequence of decreased positive selection and/or increased negative selection. Because defective positive selection is accompanied by cortical thymocyte apoptosis [158], our results are compatible with B cells and/or Ig promoting thymic positive selection. As apoptosis in the thymic cortex of μMT mice is comparable to apoptosis detected in C57BL/6 mice, and as μMT mice produce serum Ig, but few B cells [156] [157], our results suggest that serum Ig promotes thymocyte survival.

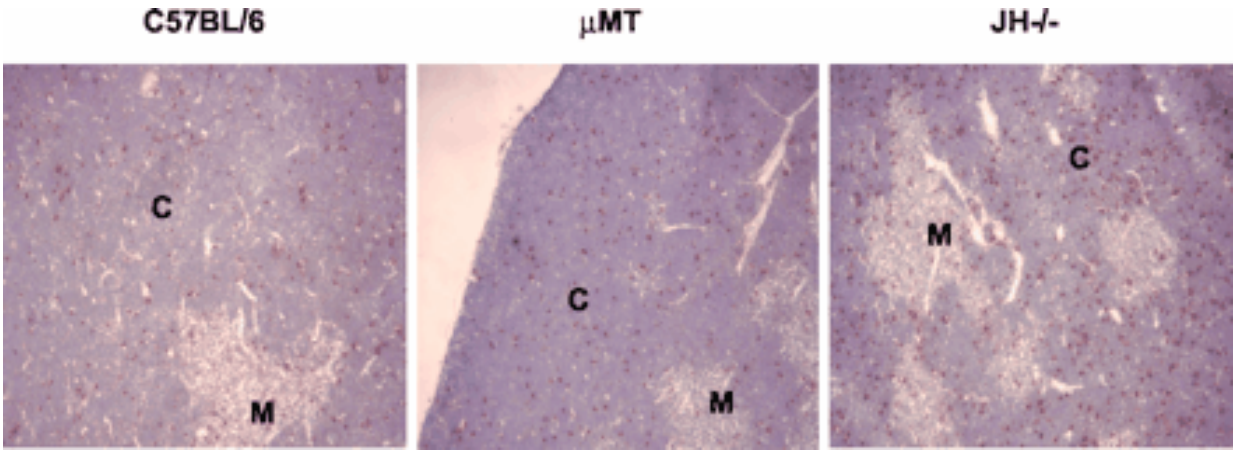


FIGURE 2. Apoptosis in the thymus. Apoptotic cells are stained brown and were detected in cryostat sections of thymi by in situ TUNEL. Photographs are representative of four mice per genotype analyzed. The number of apoptotic spots in JH-/- sections was at least 2.5-fold greater than the number of spots counted in equivalent areas of C57BL/6 or μMT sections. C, cortex; M, medulla.

4.4.3. Numbers of recent thymic emigrants are maintained and thymocyte proliferation is increased in mice that lack B cells and Immunoglobulins

We next wondered whether decreased numbers and increased apoptosis of thymocytes in JH-/- mice would change the numbers of recent thymic emigrants. Recent thymic emigrants were identified based on their tendency to take up relatively low levels of BrdU according to the method of Tough and Sprent [159, 160]. We defined the recent thymic emigrants' gate by

comparing thymectomized and nonthymectomized mice treated with BrdU. The recent thymic emigrants' gate includes the population of naive T cells lost by thymectomy (Fig. 3, *B* and *D*). Using these gates we found no differences in the proportions of recent thymic emigrants in CD4+ or CD8+ naive (CD62L-positive) T cells analyzed in JH-/-, μMT, and C57BL/6 mice, suggesting that thymic output is maintained despite decreased number of thymocytes (Fig. 3, *A* and *C*).

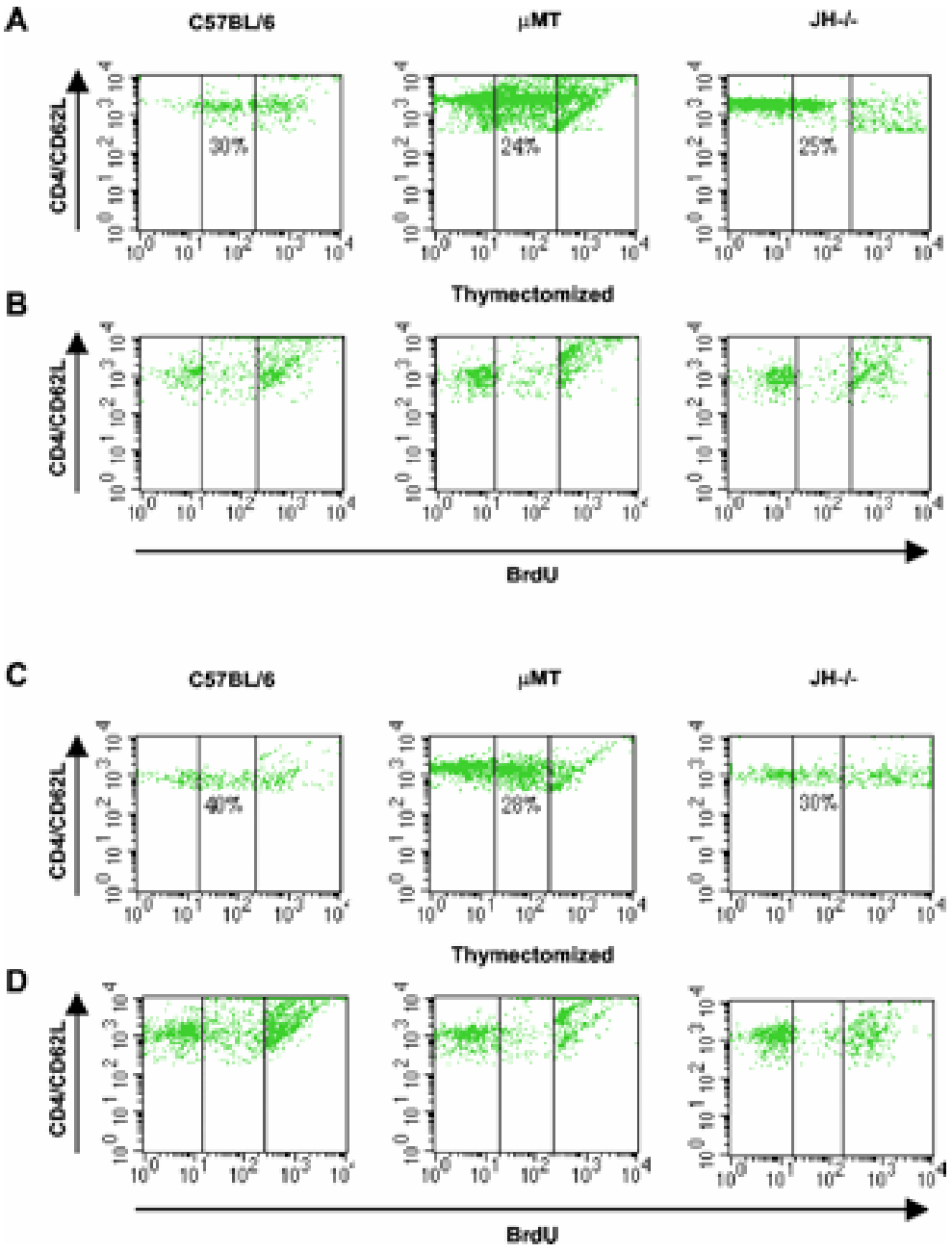


FIGURE 3. Recent thymic emigrants. The plots represent BrdU incorporation by peripheral blood CD4+CD62L+ (naive) T cells (*A* and *B*) or CD8+CD62L+ T cells (*C* and *D*). The *x*-axis shows BrdU staining fluorescence intensity; the *y*-axis shows CD62L staining fluorescence intensity. The recent thymic

emigrants are the naive CD4+ or CD8+ lymphocytes that incorporate low levels of BrdU [159, 160]. The rarity of CD4+CD62L+ and CD8+CD62L+ T cells incorporating low levels of BrdU in thymectomized mice defined the recent thymic emigrants' gates (*B* and *D*). The figure shows that thymic output did not differ significantly in C57BL/6, μMT, and JH-/- mice. The dot plots shown are representative of three independent experiments with C57BL/6 and JH-/- mice and two experiments with μMT mice.

To reconcile the decreased number of thymocytes in JH-/-mice with the maintenance of thymic output, we hypothesized that these mice have increased proliferation of thymocytes. Increased thymocyte proliferation would allow JH-/- mice to maintain thymic output despite increased cell death. Cell cycle analysis of thymocytes of JH-/-, μMT, and C57BL/6 mice revealed a 1.5-fold increase in the number of cycling (S+G2/M) thymocytes in JH-/- compared with μMT and C57BL/6 mice (Fig. 4). Our results thus suggest that lack of B cells and Ig leads to increased cell death and to thymocyte proliferation, perhaps as a compensatory mechanism to maintain T cell production.

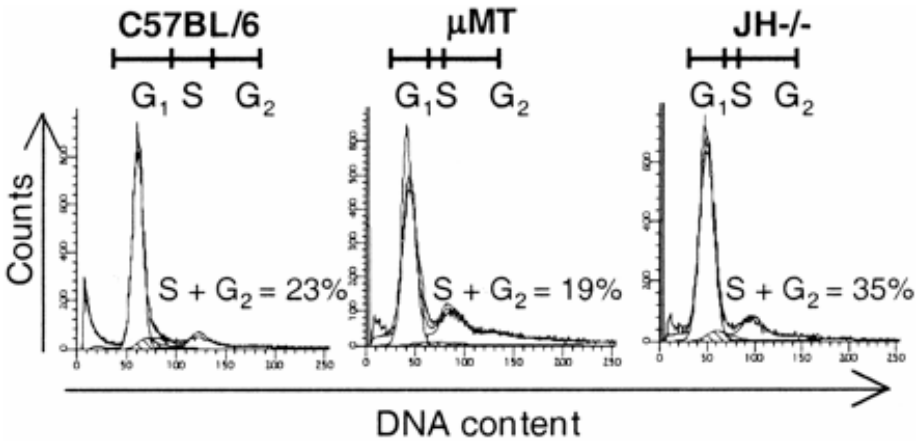


FIGURE 4. DNA content of thymocytes from C57BL/6, μMT, and JH-/- mice. Histograms of one representative experiment of a total of four per genotype are shown. The *x*-axis shows the DNA content; the *y*-axis shows the number of cells. Diagrams depict the number of cells in the G1, S, and G2/M phases of the cell cycle. The fraction of cells in the S+G2/M phase of the cell cycle (± SD) were 23 ± 0.12% for C57BL/6, 19 ± 0.14% for μMT, and 35 ± 0.19% for JH-/- mice. Twenty thousand events were collected for each analysis.

4.4.4. Contraction of the TCR repertoire in mice that lack B cells and/or Ig

If mice that lack B cells and Ig have fewer thymocytes and normal T cell egress, one might predict that the TCR repertoire would be contracted due to

clonal expansion of the fewer surviving thymocytes. As a direct test of this idea, we assayed TCR diversity in JH-/-, μMT, and C57BL/6 mice.

Until recently, quantification of TCR diversity has been accomplished by generalization from exemplary sequencing of CDR3 regions obtained from spectratyping analysis, a method that has been referred to as quantitative immunoscope analysis [161]. Although this method yielded useful estimates of TCR diversity in human and mouse [22, 24], it is only accurate as long as the number of different sequences obtained for the exemplary V(D)J joints chosen is a good estimate of the number for any other V(D)J joint. The extraordinary effort needed to perform this method in multiple individuals or mice renders it impractical for quantification of TCR diversity. These problems prompted us to develop a novel method to directly measure TCR diversity. Our method allows direct quantification of TCR diversity and was recently published [162].

To measure TCR diversity directly, we determined the number of hybridization spots (hits) of TCR Vβ-chain RNA on a gene chip (U95B; Affymetrix, Santa Clara, CA). In brief, TCR Vβ-chain cDNA is produced by RT from total RNA with a Cβ-specific primer. After second-strand synthesis, biotinylated cRNA is produced by in vitro transcription according to Affymetrix protocols and hybridized to the gene chip (U95B; Affymetrix). The diversity of the TCR Vβ-chain in a population is proportional to the number of hits above background (defined by the number of hits

corresponding to hybridization of monoclonal TCR Vβ-chain RNA) of TCR Vβ-chain-specific RNAs on the gene chip [162]. Diversity is calculated by plotting the number of hits onto a standard curve obtained for each experiment by hybridizing oligonucleotide mixtures of known diversity to individual gene chips. The number obtained varies proportionally to the actual TCR Vβ diversity, even though it does not represent the number of different TCR Vβ-chains, as each TCR Vβ-chain may generate more than one hit [162]. To determine whether the TCR Cβ-specific primer cross-hybridized with B cell RNA, we determined the diversity of cRNA obtained from purified B cells with the Cβ reverse primer. We found that the number of hits obtained from B cell cRNA prepared with the TCR Cβ-specific primer was three per 10 μg of RNA and was indistinguishable from background, indicating that the TCR Cβ-specific primer does not cross-hybridize with B cell RNA. In addition, the T cell diversity of C57BL/6 splenocytes, which include mostly B cells, was 1000-fold lower than the T cell diversity of C57BL/6 thymocytes (results not shown). Thus, the diversity of TCR Cβ-specific cRNA does not reflect contamination with B cell receptor messages.

Fig. 5 shows that the TCR Vβ diversity of JH-/- thymocytes was $6.0 \times 10^2/10$ μg of RNA compared to that of wild type, which was $1.1 \times 10^8/10$ μg of RNA ($p = 0.0002$). By showing profoundly decreased thymocyte diversity in mice that lack B cells and Ig (JH-/-), our results indicate that B cells and/or Ig promote thymocyte diversity. In this experiment we compared age-matched

JH-/- and C57BL/6 mice that were between 5 and 12 wk old. The decreased thymocyte diversity in JH-/- mice was thus not due to age-dependent atrophy of the thymus.

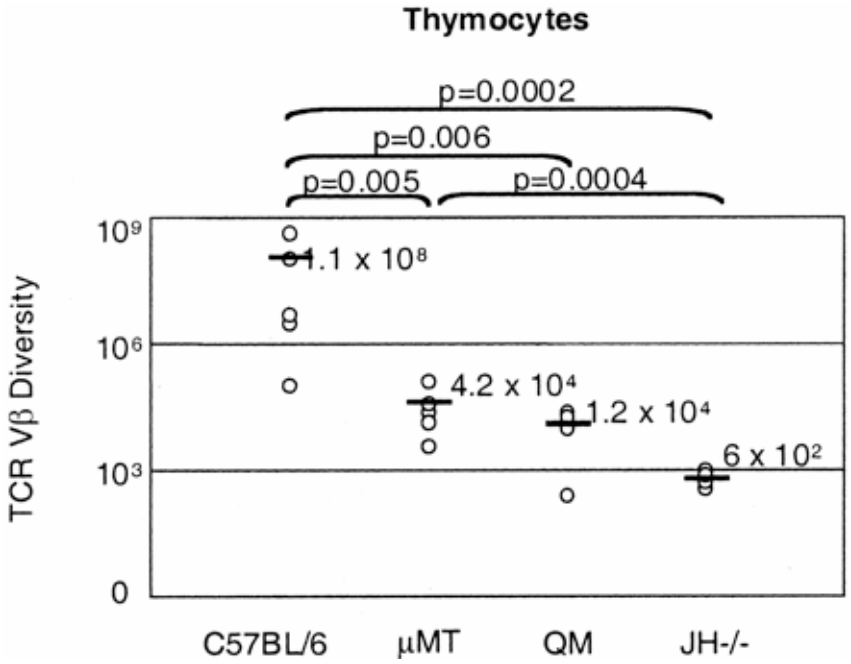


FIGURE 5. TCR Vβ diversity. *A*, TCR Vβ diversity of thymocytes obtained from C57BL/6, μMT, and JH-/- mice. The *x*-axis shows mouse strains; the *y*-axis shows TCR Vβ diversity. Thymocytes were obtained from 5- to 12-wk-old mice as previously described [129], and mononuclear cells were isolated on Ficoll-Paque gradient (Sigma-Aldrich). Total RNA was obtained with the RNeasy kit (Qiagen). First-strand cDNA was synthesized with a reverse primer containing a T7 polymerase promoter 3' overhang that annealed to

the TCR constant region, with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The second strand was generated by nick translation. The double-strand cDNA was processed for gene chip hybridization according to Affymetrix protocols. Hybridization hits were summed, and the total number was used to calculate TCR Vβ diversity by comparison with known diversity oligomer mixtures (standards) run at the same time. Measurement of TCR Vβ diversity was specific, because when it was applied to purified diverse B cells (with <0.9% of T cells), it yielded background values (three per 10 μg of RNA). Each open circle represents one experiment, and the values indicate the mean of TCR Vβ diversity for each strain of mice. Statistical analysis was performed on each of the log-transformed numeric values by unpaired two-sided *t* test.

Next, we asked whether Ig could promote T cell diversity under the condition of B cell deficiency. To this end we analyzed the TCR Vβ diversity in thymocytes of μMT mice that have serum Ig, but very few B cells. We found that TCR Vβ diversity in thymocytes of μMT mice was 4.2×10^4/10 μg of RNA and 70-fold greater than TCR Vβ diversity in thymocytes of JH-/- age-matched mice (6.0×10^2/10 μg of RNA; $p = 0.0004$). Our results indicate that Ig and/or the few B cells in μMT mice contribute to T cell diversity. The reduced TCR diversity in B cell-deficient mice was maintained in the periphery as JH-/-, and μMT mice splenocytes had 1000- and 10-fold reduced TCR Vβ diversity, respectively, compared with wild-type mice.

Selection of a diverse T cell repertoire requires TCR recognition of diverse self-peptides in the context of self-MHC [140]. Because the V regions of H and L chains of antibodies are a potential source of diverse self-peptides, we wondered whether T cell diversity depended on the diversity of the B cells. To address this question, we determined TCR Vβ-chain diversity of thymocytes obtained from QM mice that have 80% of B cells from a single clone and polyclonal serum Ig [129, 130]. In QM mice, the diversity of the JH4-containing H chains is only 0.01% that in wild-type [162]. Fig. 5 shows that the TCR Vβ diversity of QM thymocytes was, on the average, 1.2 x 10^4/10 μg of RNA, not significantly different from that of μMT and 0.01% that of WT thymocyte diversity (p = 0.006). Our findings of comparable TCR diversity in QM and μMT age-matched mice indicate that oligoclonal B cells do not promote diversification of T cells.

4.4.5. B cell precursors in C57BL/6, QM, JH-/- and μMT thymi

Our results imply that B cells and/or Ig promote the selection of a diverse T cell repertoire, presumably in the thymus. As both mature B cells and B cell precursors are found in the thymus [163, 164], we wondered which B cell populations were present in the thymus of C57BL/6, QM, JH-/-, and μMT mice. Fig. 6A shows that JH-/- and μMT thymi had fewer CD19+ cells in the thymic cortex and fewer CD19+ and B220+ cells in the medulla compared with C57BL/6 mice. In contrast, the numbers of B220+ cells were

comparable in the thymic cortexes of JH-/-, μMT, and C57BL/6 mice (Fig. 6A). To discriminate B cell precursors from mature B cells, we analyzed thymocytes of JH-/-, μMT, QM, and C57BL/6 mice by flow cytometry. Fig. 6B shows that mature B cells (IgM+ and B220+) are missing from JH-/- and μMT thymi and are reduced by half in QM thymi compared with those in C57BL/6 thymi. There were very few pre-B cells (IgM-CD43-B220+) [165] in the thymi of mice of all genotypes, whereas they were present in the bone marrow (Fig. 6C). Pro-B cells (IgM-CD43+B220+) [165] did not differ significantly in JH-/-, μMT, QM, or C57BL/6 thymi (Fig. 6C). Our findings thus suggest that mature B cells, rather than B cell precursors, promote thymocyte selection and the generation of T cell diversity.

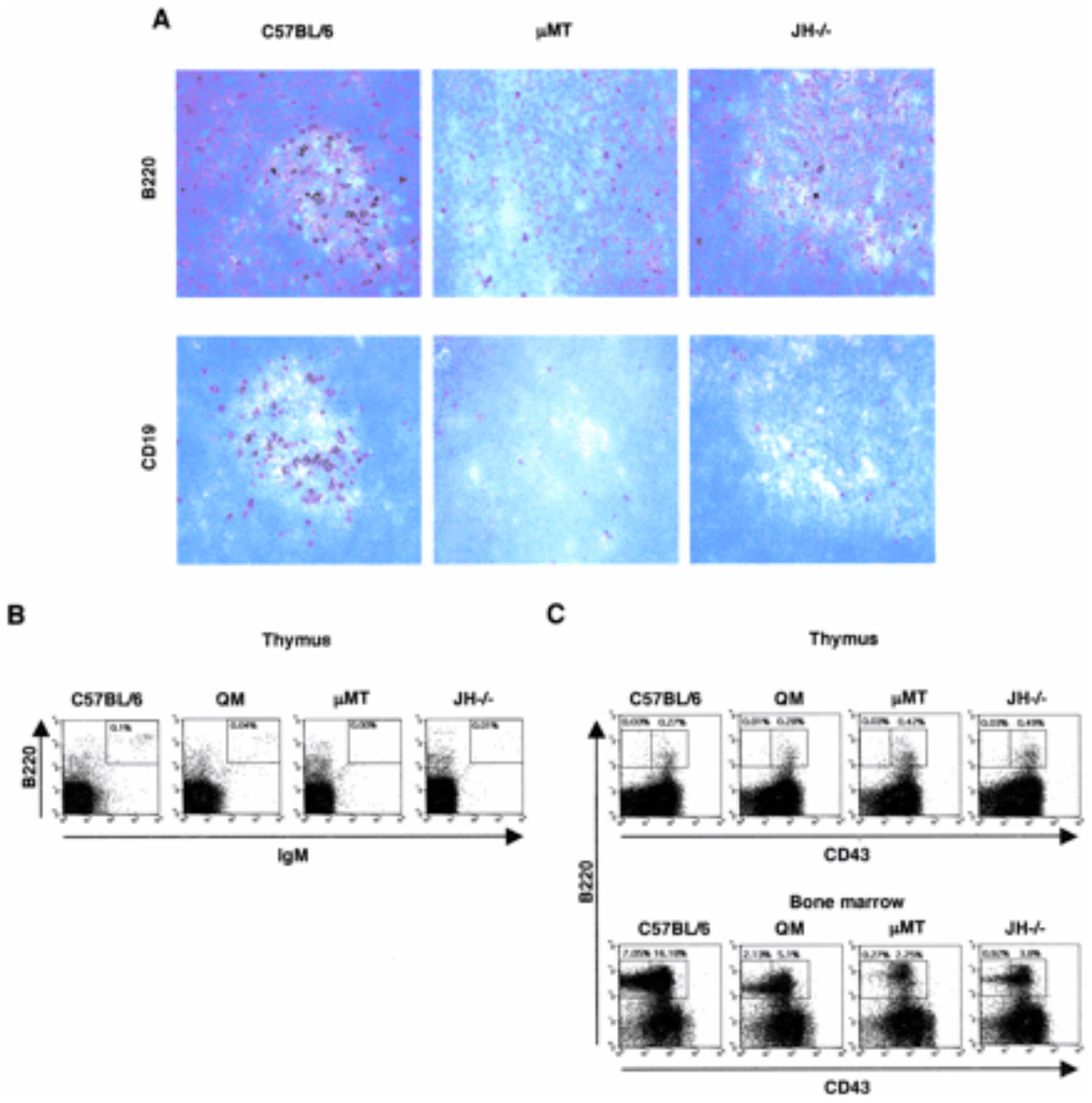


FIGURE 6. Immunohistochemical and flow cytometric analyses of thymic B cells and B cell precursors. A, Immunohistochemical staining of thymic sections of C57BL/6, µMT, and JH-/- mice. Positive cells are stained brown. JH-/- and µMT thymi had fewer CD19+ cells in the thymic cortex and fewer CD19+ and B220+ cells in the medulla compared with C57BL/6 mice. The numbers of B220+ cells were comparable in the thymic cortexes of JH-/-,

μMT, and C57BL/6 mice. B, Flow cytometric analysis of mature B cells in the thymi of C57BL/6, QM, μMT, and JH-/- mice. The x-axis shows IgM staining fluorescence intensity; the y-axis shows B220 staining fluorescence intensity. The dot-plot diagrams identify mature thymic B cells that are B220+ and IgM+. Mature B cells are missing from the thymi of μMT and JH-/-, but are present in the thymi of C57BL/6 and QM mice. Percentages represent the proportion of thymocytes that are mature B cells. The results shown are representative of three mice per genotype that were between 6 and 10 wk of age. C, Flow cytometric analysis of pro- and pre-B cells in the thymi or bone marrow of C57BL/6, QM, μMT, and JH-/- mice. The x-axis shows the CD43 staining fluorescence intensity; the y-axis shows the B220 staining fluorescence intensity. The plots represent IgM- cells. Upper diagrams represent thymocytes; lower diagrams show bone marrow cells. There were no significant differences in the proportions of pre-B cells (IgM-CD43-B220+) and pro-B cells (IgM-CD43+B220+) in JH-/-, μMT, QM, or C57BL/6 thymi. The percentages refer to the fraction of IgM- cells that corresponds to the indicated phenotype. Pre-B cells were nearly absent in the thymi of mice of all genotypes, whereas they were present in the bone marrow.

4.4.6. T cell diversity in JH-/- mice reconstituted by adoptive transfer of B cells or administration of immunoglobulin.

Next we tested whether providing B cells and/or immunoglobulin could increase TCR diversity in JH-/- mice lacking both B cells and immunoglobulin. To this end, we injected newborn JH-/- mice with bone marrow-derived wild type or monoclonal B cells, or with polyclonal or monoclonal IgG, and measured TCR diversity after 4 weeks. The presence of adoptively

transferred B cells was verified by flow cytometry analysis; recipient mice had between 10% and 20% of B cells in peripheral blood lymphocytes, at the time of sacrifice (unpublished data). Mice injected with immunoglobulin had, on average, serum concentrations greater by 4.7 fold than wild type at the time of sacrifice (table I and unpublished data).

Transfer of wild type B cells in JH-/- mice increased thymocyte TCR Vβ diversity by 8-fold; however, adoptive transfer of monoclonal B cells did not, indicating that B cell diversity is required for the generation of thymocyte diversity. Similarly, injection of polyclonal IgG in JH-/- mice increased thymocyte TCR Vβ diversity by 7-fold, while injection of monoclonal IgG or ovalbumin did not (table II).

Table 2. Reconstitution of TCR diversity in JH-/- mice after adoptive transfer of bone marrow B cells or administration of IgG.

JH-/- recipient	**TCR diversity of thymocytes**	**Variation from JH-/-**
Not reconstituted	6.5×10^2	1
Reconstituted with Polyclonal IgG	4.3×10^3	6.6
Reconstituted with Polyclonal B cells	5.4×10^3	8.3
Reconstituted with Monoclonal IgG	1.9×10^1	0.03
Reconstituted with Monoclonal B cells	5.3×10^2	0.8
Reconstituted with Ovalbumin	1.6×10^2	0.25

These results show that diverse immunoglobulin promotes TCR Vβ diversity. In agreement with this function for immunoglobulin in promoting T cell diversity, μMT mice with serum immunoglobulin that is 4.8% of wild type, had 70 fold greater T cell diversity than JH-/- mice that had no serum immunoglobulin (table I).

Since injection of polyclonal IgG alone increases TCR diversity, we wondered whether production of immunoglobulin was the mechanism by which adoptively transferred B cells promoted T cell diversity. To find out, we

quantified serum immunoglobulin in mice recipients of wild type B cells, 4 weeks or later post-transfer. Table I shows that JH-/- mice with adoptively transferred B cells had no measurable serum immunoglobulin, while JH-/- mice injected with polyclonal IgG had levels of immunoglobulin in the serum that were 4.8 fold greater than wild type. One possible conclusion is that polyclonal B cells promote TCR diversification in the absence of serum immunoglobulin but we cannot exclude the possibility that antibodies transiently present in the serum promoted TCR diversification.

4.5. Discussion

Here we report that diverse B cells and polyclonal immunoglobulin drive the selection of a diverse repertoire of T cells. This finding questions the long-standing idea that T and B cells develop independently [8]. Generation of a diverse TCR repertoire relies on the quasi-random recombination of the TCR V region gene segments and on positive and negative selection [140]. Positive selection and MHC restriction are generally thought to depend on interaction of T cell precursors with thymic epithelial cells [166]; however, this concept is at odds with experimental evidence indicating that mice bearing T cells and thymic epithelial cells that are haplo-incompatible are also restricted to the MHC of nonthymic epithelial cells [147, 167, 168]. These observations could suggest that B cells colonizing the thymus promote thymocyte selection and MHC restriction.

Our results showing reduced TCR diversity in mice that lack B cells (JH-/-) or that have very few B cells (μMT) could be explained if B cells promoted positive selection of thymocytes by decreasing death by neglect. In agreement with the concept that B cells promote positive selection is the finding of a decreased number of thymocytes and increased cell death in the thymic cortex of mice that lack B cells. We cannot, however, rule out the possibility that B cells decrease negative selection of thymocytes. Against this possibility are reports that thymic B cells promote, rather than decrease,

negative selection of T cells to murine mammary tumor virus, minor lymphocyte-stimulating antigens [169], and I-E expressed only on B cells [170]. Another possibility is that B cells may promote colonization of the thymus by thymic precursors or early thymocyte development before the expression of TCR and thus before selection.

As selection of a diverse T cell repertoire in the thymus is thought to depend at least in part on the diversity of peptides presented by self-MHC [140], we hypothesized that B cells and Ig may provide an alternative source of diverse peptides derived from the V region of Ig or from captured peripheral antigens. We tested this hypothesis in mice with QM B cells that are 1000-fold less diverse than wild-type B cells [162] and have normal numbers of T cells (figure 8 of this thesis). Our finding of severely reduced T cell diversity in QM mice indicates that diversity of B cells may be more important than the number of B cells in the generation of a diverse T cell repertoire.

Our results showing increased diversity of T cells after injection of polyclonal IgG indicate that some of the effects of B cells in the selection of T cells can be mediated by secreted Ig. However, adoptive transfer of polyclonal B cells also increased TCR diversity in JH-/- mice in the absence of detectable serum Ig (Tables II and I). One possible interpretation is that polyclonal B cells promote TCR diversification in the absence of serum Ig, but we cannot exclude that antigens present transiently in the serum may have contributed

to the increased T cell diversity following adoptive transfer of B cells. Thus, whether B cells may contribute to a mature T cell repertoire by means other than by producing Ig is not known.

Thymic epithelial cells may produce some of the peptides required for positive selection, but these may not be diverse enough to assure the survival of thymocytes representing wild-type TCR diversity. Consistent with the requirement that diverse self antigens be presented to developing thymocytes by stromal cells, it was recently showed that medullary thymic epithelial cells (MECs) express a number of genes coding for ectopic proteins. Autoimmune regulator (AIRE) is a nuclear protein identified as the regulator of this process promoting central tolerance by negative selection of autoreactive thymocytes [27]. The AIRE protein contains several domains that function as a nuclear transcription factor, interacting with multiple components of the transcriptional complex [171]. AIRE exerts its function in a dosage-dependent manner. This way a slight decrease in AIRE gene function may decrease thymic protein expression, allowing delivery of autoreactive T cell clones in the periphery. Mice that lack expression of AIRE develop autoimmunity but have normal number of thymocytes, normal CD4/CD8 subset distributions and spleen and lymph node cell numbers [27, 172]. Thus, the role of AIRE shaping the T cell repertoire occurs through negative selection and purging of autoreactive cells from the thymocyte repertoire.

By means of AIRE-mediated negative selection the immune system must avoid aggressive T cell responses against self-antigens. But, paradoxically, exposure to self-peptides seems to have an important role in positive selection in the thymus and the maintenance of a broad T-cell repertoire in the periphery. T cell precursor's selection in the thymus seems to depend on deleting mechanisms, as negative selection, and on non-deleting mechanisms, which help to shape the T cell repertoire. Non-deleting mechanisms may include processes that balance and adjust antigen density and T cell avidity, promoting survival of thymocytes. This phenomenon is recognized as 'tuning' of the T cell development process and may maximize the peripheral T-cell repertoire by allowing the survival of T cells that can respond to self.

The 'tuning' process may occur when the density of peptide-MHC complex is below that required to induce deletion and is not optimal, immediately leading to positive selection. Hypothesis of how the 'tuning' may occur relates with: 1) alteration of antigen processing by APC in the thymus (changing the peptide density on APC); 2) alteration of the T cell avidity for a single self peptide-MHC complex, for example, through alter peptide ligands or the presence of co-receptores or adhesion molecules that affect thymocyte-stromal cell avidity; 3) alteration of key elements in TCR signaling pathways that would change T cell activation and survival trigger.

Immunoglobulins are self, highly diverse molecules constantly present in the thymus. This work shows that Ig molecules are used to increase TCR

repertoire. The effect of Ig peptides promoting TCR diversification may possibly be explained by the effect of presentation of highly diverse self-peptides in the thymus (Ig peptides) during the process of T cell development. Furthermore, because of similarities on TCR and Ig molecules structure [173, 174], a possible preference of the TCR to Ig-MHC complex may be present, facilitating the role of Ig as promoter of positive selection and T cell repertoire diversification.

The mechanisms of TCR-based selection prevent autoimmunity by maintaining the avidity of the normal self-reactive T-cell repertoire at a low level. This way, it assures protection against producing a repertoire which, in this case, could likely recognize Ig molecules with intermediate to high affinity. These mechanisms would buffer excessive autoreactivity while optimizing TCR repertoire usage. The activation of possible self-reactive T cells in the periphery would, therefore, require levels of self-peptide–MHC complexes that might not be achieved under physiological conditions in the thymus. One hypothesis is that the amount of Ig peptides in the thymus is sufficient to activate positive selection via low avidity interactions but the same amount of Ig peptides is not sufficient to activate peripheral T cells

Activation of a pathological autoreactive response would, therefore, require a combination of factors that may increase the level of self-antigen that is available. These states seem to be regulated both by positive and negative selections, as very restrict TCR repertoires are related to autoimmunity [175].

Our findings reveal a heretofore unrecognized and vital function of B cells and Ig, in promoting TCR diversification, perhaps by contributing to the diversity of the peptides that developing T cells encounter. This function of B cells may help to explain the phenotype of B cell-deficient human subjects and suggests a potential approach to immune reconstitution.

CHAPTER V

Immunoglobulin promotes the diversity and the function of T cells

Immunoglobulin promotes the diversity and the function of T cells

Cristina João[1 2 *], MD, Brenda M. Ogle[3], PhD, Susan Geyer, PhD[4]

[1] Internal Medicine Department, Division of Hematology, Mayo Clinic, Rochester, MN, USA*; [2] Hematology Department, Portuguese Institute of Oncology, Lisbon, Portugal; [3] Transplantation Biology Unit, Surgery Department, Mayo Clinic, MN, USA; [4] Division of Biostatistics, Mayo Clinic College of Medicine, Rochester, MN, USA

This study was funded by the grants from the National Institutes of Health (AI48602, AI41570, HL46810) and from Fundação para a Ciência e Tecnologia, Lisbon, Portugal (BD 6797/2001).

European Journal of Immunology 2006, 36:1718-1728

5.1. Abstract

It is generally accepted in Immunology that while T- and B-cells collaborate for the production of antibodies in response to protein antigens, T-cells develop and function in the absence of B-cells. However B-cell deficient subjects and mice have unexplained cellular immune defects. Here we examined the contribution of B-cells/Ig for the generation of diversity and function of T-cells. Mice lacking B-cells and Ig (JH-/-) or having oligoclonal B-cells (QM) had a profoundly contracted T-cell receptor (TCR) Vβ repertoire: 0.08% and 1.3% of wild type, respectively. Rejection of H-Y incompatible skin graft in QM and JH-/- mice was significantly delayed (median, 43 and 22 days, respectively) compared to wild type mice (median, 16 days). Furthermore, reduction of TCR V β diversity by thymectomy in wild type mice significantly increased survival of H-Y incompatible skin grafts and reconstitution of T-cell diversity in QM mice with immunoglobulin Fab fragments significantly decreased survival of the skin grafts. These results indicate that B-cells and/or Ig "help" T-cells through the generation and maintenance of T-cell diversity, improving T-cell function. Our results may have important implications on therapy and immune reconstitution in the context of AIDS, cancer, autoimmunity and post myeloablative treatments.

Keywords: TCR repertoire, immunoglobulin, T cell function, immune reconstitution.

5.2. Introduction

It is generally accepted in immunology that T and B cell compartments develop separately. The concept of two lymphoid compartments originated from studies in the chicken that established that there were "at least two basic levels of immune responses related functionally to different primary lymphoid organs", the thymus and the bursa of Fabricius [8]. One level requiring thymus integrity concerned allograft rejection and graft-versus host disease while the other, requiring the bursa, was necessary for antibody production [8]. Thus bursectomy did not changed cellular immune responses such as allograft rejection [4, 8] and thymectomy, causing defective cellular immune reactions, maintain plasma cells and circulating immunoglobin [4]. However, thymectomized birds [4] and thymectomized newborn mice [176] had impaired antibody responses to antigens, thus contradicting the first concept of two independent lineages. This contradiction was resolved by the discovery of T cell "help" for the production of antibodies [177, 178].

Apart from development, the separation between the functions of B and T cells is no longer accepted. However, the extent and the mechanisms by which B cells "help" T cells is today a matter of controversy. In B cell deficient mice several T cell responses to microorganisms such as *Salmonella enterica* [179], *Francisella tularensis* [180], *Plasmodium chabaudi chabaudi*

[181], *Chlamydia trachomatis* [182] are compromised. Also, patients with X-linked agammaglobulinemia (Bruton's syndrome) who don't have peripheral B cells and immunoglobulin and are often said to have normal cell mediated immune responses, have an increased risk of developing arthritis and respond sub-optimally or not at all to recall antigens in tests of delayed-type hypersensitivity [183] suggesting deficient T cell function secondary to the lack of B cells and immunoglobulin.

Several mechanisms have been proposed whereby B cells may modify T cell functions. Those include B cell antigen presentation [100], production of antibody which may enhance [101] or depress [102] cellular immunity, B cell dependent development of follicular dendritic cells in peripheral lymphoid organs [184], and effects on regulatory T cells [104]. We proposed recently that polyclonal B cells and/or immunoglobulin contribute to development of thymocyte diversity possibly by mechanisms related to antigen presentation of highly diverse peptides originated from Ig [185]. In this paper we tested whether B cells and/or immunoglobulin contribute to the diversity of peripheral T cells and whether B cell-dependent T cell diversity influences H-Y incompatible skin allograft rejection as an example of T cell function. We compared wild type mice with mice that lack B cells and Ig (JH knockout mice) and mice that have oligoclonal B cells (QM mice). All these strains of mice are in the same genetic background, H-2b.

5.3. Materials and Methods

5.3.1. Mice. The B cell-deficient strains used were the JH-/- mice that lack mature B cells and immunoglobulin [131] and the Quasimonoclonal mouse (QM) where 80% of the B cells are monoclonal [129]. C57BL/6 mice were purchased from the Jackson Laboratories. JH-/- and QM mice were bred and all mice were housed in a specific pathogen-free facility at the Mayo Clinic. All mice were between 0 and 20 weeks of age and all animal experiments were carried out in accordance with protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee.

5.3.2. Determination of TCRVβ diversity

Isolation of RNA. Spleens harvested from mice were placed in RPMI and pushed through a 70 μm cell strainer. Lymphocytes were isolated by Ficoll-paque (Amersham Biosciences, Piscatanaway, New Jersey) gradient. Total RNA was obtained with Qiagen RNeasy kit (Qiagen, Inc., Valencia, California) per the manufacturer's instructions.

Generation of diversity standards. Diversity standards were prepared through the generation of oligonucleotide mixtures of known diversity.

For example, to generate a standard diversity of 10^6, 18-mer oligonucleotides were synthesized with 10 sites of random assignment generating 4^{10} = 1,040,526 different oligomeres. Similarly, we created oligomer mixtures with 1, 10^3 and 10^9 variants. Oligonucleotides were biotin-labeled and hybridized to the gene chips as explained below.

Generation of lymphocyte receptor-specific cRNA. First strand cDNA was obtained by reverse transcription with a mouse TCR Cβ reverse primer:

*T7+C*β (5'-

GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGCTTGGGTGGA

GTCACATTTCTC - 3'). Second strand synthesis and preparation of biotin-labeled cRNA was conducted according to Affymetrix standard protocols (Affymetrix, Inc., Santa Clara, California).

Application of cRNA to the gene chip. Equal amounts of cRNA from different samples and diversity standards were hybridized to U133B gene chips (Affymetrix, Inc., Santa Clara, California). Gene chips were processed at the Microarray Core Facility, Mayo Clinic, Rochester, MN.

Data analysis. For each gene chip experiment, we obtained raw data corresponding to oligo location and hybridization intensity. Data were arranged in order of ascending hybridization intensity. The number of oligo locations with intensity above background (i.e., number of hits) was summed. The standard curve was generated from hybridization of samples with known numbers of different oligomeres. The standard oligonucleotide

mixtures were 18-mer oligomeres synthesized to obtain mixtures containing 1, 10^3, 10^6 and 10^9 different oligonucleotides. Diversity of the test samples was extrapolated from the standard curve. This assay specifically detects TCR Vβ diversity since sorted B cells from C57BL/6 splenocytes containing 0.09% CD3 positive cells yielded a mean diversity of 136 (standard deviation of 89) corresponding to 0.023% of the diversity obtained from equal numbers of splenocytes containing 20% CD3 positive cells.

5.3.3. Vβ amplification and sequencing. Total RNA isolated from C57BL/6 and JH-/- mice splenocytes (RNeasy mini kit, Qiagen Inc., Valencia, CA, USA) was reverse transcribed into cDNA using a ThermoScript™ RT-PCR System (Invitrogen Corporation, USA) according to the manufacture's instructions. The following PCR reaction was done with 2 µl of cDNA from each sample, 39.5 µl sterile water, 5 µl 10 x cloned *Pfu* DNA polymerase reaction buffer (Stratagene, CA, USA), 10 pmol of Vβ 8.1-specific primer (forward) (CATTACGCATATGTCGCTGAC) in a 1 µl vol., 10 pmol of Cß (reverse) (GAGACCTTGGGTGGAGTCAC) in a 1 µl vol. and 1.25 U *Pfu* Turbo DNA polymerase (0.5 µl; Stratagene, CA, USA). The PCR conditions were 94°C, 30 seconds; 60°C, 30 seconds; and 72°C, 1 min for 35 cycles, followed by a final extension of 7 min. The presence of specific PCR products was confirmed by size bands on a 1% agarose gel and the PCR products were

cloned using TOPO TA cloning (Invitrogen Corporation, USA) according to the manufacture's instructions. Sequencing was performed on an ABI PRISM™ 377 DNA Sequencer at Mayo Clinic Molecular Biology Core Facility using the TOPO TA vector primers. Analysis of the sequences was done using the Sequencher™ (Gene Codes Inc.) software.

5.3.4. FACS analysis. Splenocytes were obtained by mincing spleens through a 0.70 μm mesh followed by hemolysis in a NH_4Cl buffer. Total splenocyte numbers were counted with a Coulter counter. Cells were stained with one, two or three of the following monoclonal antibodies (all the antibodies were from BD Pharmingen) as described [129]. Fluorescein isothyocyanate (FITC)-conjugated: rat anti-mouse CD4 (GK 1.5), rat anti mouse CD8α (Ly-2); phycoerythrin (PE)-conjugated: rat anti-mouse CD8α (53-6.7), rat anti mouse CD44 (Pgp-1, Ly-24), rat anti mouse CD25 (PC61); and biotin-conjugated: rat anti-mouse CD3ε (145-2C11). Lymphocytes were gated on the light scatter plot by back gating onto $CD4^+CD3^+$and $CD8^+$ $CD3^+$ cells; numbers of the splenocytes sub-populations were determined by multiplying the percentage as defined by gating on the FACS plot, by their total number.

5.3.5. Proliferation assays. $CD4^+$ splenocytes were purified using an automated magnetic activated cell sorter (AutoMACS) system ($CD4^+$ T cells isolation kit, Miltenyi Biotec, Auburn, CA). 2 $x10^5$ $CD4^+$ T cells were cultured for 4 days in round bottom, 96-well culture plates with 10 μg/ml of anti CD3ε (clone 145-2C11, eBiosciences, San Diego, CA) and 10 μg/ml of Anti CD28 (clone 37-51, BD Pharmingen, San Diego, CA) or with purified B cells (CD19+ cells isolation kit, Miltenyi Biotec, Auburn, CA) in the presence of several concentrations of OVA 323-329 peptide. In the case of activation with CD3ε/CD28, after being coated for 3 h at 37° C with anti CD3 ε the wells were washed 3 times with cold PBS and the cells incubated with soluble anti CD28. Incorporation of [^{3}H]thymidine (1 μCi/well) by proliferating cells was measured during the last 16 h of culture.

5.3.6. Skin Grafts. Skin grafts were performed according to a modified technique of Billingham et al. [186]. Briefly, full thickness tail skin (1 x 1 cm) was grafted on the lateral flank. Grafts were observed daily after removal of the bandage at day 8 and considered rejected when 90% or more of the graft was shed off from the graft bed.

5.3.7. Thymectomy. The thymus was exposed by a median sternotomy to the second anterior rib, the connective tissue dissected and the thymic lobes

removed by suction. The muscles overlying the trachea and the skin were sutured. The accomplishment of total thymectomy was confirmed for all the animals at the time of sacrifice by inspection of the thorax.

5.3.8. Reconstitution: Mice injections. Mice were weekly injected *i.p.* with 250 μg of immunoglobulin Fab fragments or mouse polyclonal IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) since birth to graft rejection.

5.3.9. Statistical analysis. Data for each of the immunologic parameters of interest were summarized for each of the mouse strains. Differences in these measures were explored graphically in addition to their descriptive summarization. Where appropriate, comparisons of median measures between mouse strain cohorts was limited to nonparametric evaluations; e.g. the Wilcoxon rank sum test for comparing medians between two mouse strains. Time to graft failure was graphically evaluated using the methods of Kaplan and Meier. Differences in the time to graft failure distributions were assessed in an exploratory manner using log rank statistics. Statistical significance was declared for p-values < 0.05.

5.4. Results

5.4.1. B cell deficient mice and mice with quasi-monoclonal B cells have a severely contracted peripheral T cell receptor repertoire

To study the role of B cells and immunoglobulin in the establishment and maintenance of the peripheral T cell repertoire, we took advantage of several B cell deficient mice. We found previously that B cell deficient mice (JH-/- [131] and QM mice [129] have profoundly contracted TCR diversity of thymocytes [185]. To determine the extent to which these mice also had contracted peripheral T cell diversity, we measured TCR diversity in splenocytes of JH-/-, QM and wild type mice according to Ogle et al. [162]. As reflected in Figure 1, the average TCR Vβ diversity of JH-/- splenocytes (mean = 482.8 per 10 μg of RNA) was substantially lower than that of the wild type (mean = 603997.3 per 10 μg of RNA). In addition, we found that JH-/- mice have lower numbers of splenic T cells (Figure 2A and [108]) as well as defective peripheral lymphoid organogenesis with absent follicular dendritic cell network (Figure 2B and [109]).

To determine whether B cells and/or B cell products (Ig) contribute to the diversity of T cells in the presence of normal secondary lymphoid organs and similar numbers of CD3+ T cells, we measured TCR diversity on splenocytes of QM mice. QM mice have quasi-monoclonal B cells and normal levels of immunoglobulin [185, 187], had normal secondary lymphoid structure with a

mature follicular dendritic cell network (Figure 2B) and normal number of splenic T cells (Figure 2A), contrary to what occurs on the JH-/- mice model.

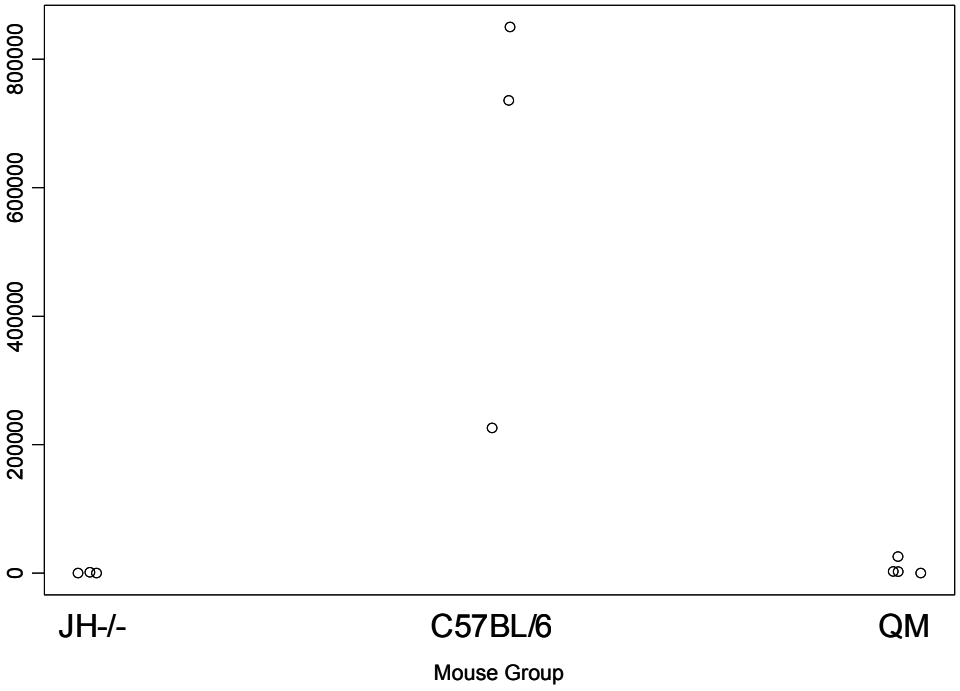


Figure 1 – TCR Vβ diversity quantified in splenocytes. Were studied 3 mice lacking B cells and immunoglobulin (JH-/), 4 mice with oligoclonal B cells (QM) and 3 wild type mice (C57BL/6). All the mice were age-matched.

Figure 2A

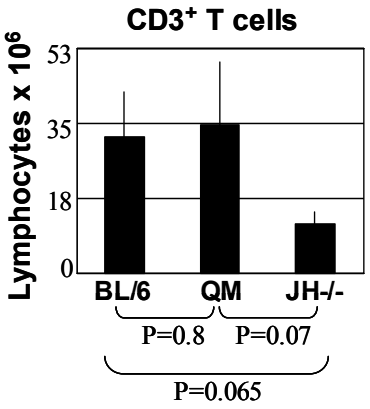


Figure 2B

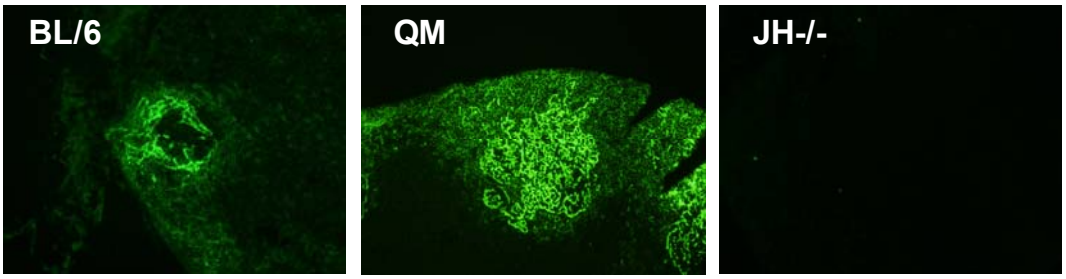


Figure 2 – Number of T cells (A) and presence of follicular dendritic cells network in lymph nodes (B) in C57BL/6, QM and JH-/- mice. A - The numbers of T cells were calculated by multiplying the respective percentage of the total events as defined in the flow cytometry dot plot analysis with specific CD3 monoclonal antibodies, by the total number of WBC obtained by counting on a Coulter. The number of $CD3^+$ splenocytes (average ± SD) was $3.2 \times 10^7 \pm 3.0 \times 10^7$ in C57BL/6 (n=8), $3.5 \times 10^7 \pm 2.9 \times 10^7$ in QM (n=4) and $9.1 \times 10^6 \pm 7.7 \times 10^6$ in JH-/- mice (n=6). Error bars represent standard deviation. Mice were between 5 and 20 weeks of age.

Comparisons between each two groups of mice (p values indicated below the diagrams) were calculated by the Wilcoxon test.

B - Follicular dendritic cells network are stained fluorescent green and were detected in cryostat sections of lymph nodes using staining with monoclonal antibody directed against murine CD21/CD35. Photographs are representative of three to four different mice per genotype analyzed.

Similar to what seen for splenocytes of JH-/-, the TCR Vβ diversity of QM splenocytes was substantially lower than that for those of wild type (QM mean = 7683.2 vs. 603997.3 per 10 μg of RNA, respectively) (Figure 1). Because contracted T cell diversity occurs in mice with normal lymphoid organs (QM mice) and does not reflect differences in T cell number as QM mice have normal numbers of CD3+ T cells, we conclude that the number of T cells is not a confounder in the association between peripheral TCR diversity and the presence of polyclonal B cells or immunoglobulin. Severely reduced TCR diversity in QM mice that are B cell and immunoglobulin proficient indicate that T cell diversity is not a function of B cell number and/or of serum Ig concentration [185] but may rather depend on the diversity of B cells and immunoglobulin in the serum, i.e., on the polyclonality of the B cells/Ig compartment.

5.4.2. Oligoclonal expansion on JH-/- T cells

To examine whether contraction of the T cell repertoire in the JH-/- mice was accompanied by oligoclonal expansion we analyzed the joining sequences of TCR variable exons containing the $V\beta_{8.1}$ gene. To this end we amplified, cloned and sequenced 46 and 59 of TCR$V\beta_{8.1}$ rearrangements from cDNA obtained from wild type and JH-/- splenocytes, respectively. We found no preferential Jβ segment usage in JH-/- or C57BL/6 mice and that all variable exon genes sharing $V\beta_{8.1}$ and $J\beta_{2.6}$ or $J\beta_{2.1}$ (these Jβ segments were the most frequent chosen, 10 and 15 for JH-/- and 8 and 7 for C57BL/6, respectively) had different VDJ joining sequences and do not show evidence of preferential clonal expansion (Table 1 and 2). Although, clonal expansion of other Vβ families besides $V\beta_{8.1}$ cannot be excluded, the extreme contraction of TCR repertoire in JH-/- mice seems to be a consequence of gaps or skewness in the repertoire.

Table 1 – Number of Jβ families represented in the cloned and sequenced TCR Vβ 8.1 – Cβ region in C57BL/6 and JH-/- mice. The sequences of the Jβ families were taken from the literature [21, 188].

	BL/6 46 sequences	**JH-/- 59 sequences**
Jβ 1.1	0	3
Jβ 1.2	5	8
Jβ 1.3	0	1
Jβ 1.4	2	1
Jβ 1.5	3	0
Jβ 1.6	1	1
Jβ 2.1	7	8
Jβ 2.2	4	6
Jβ 2.3	3	8
Jβ 2.4	4	8
Jβ 2.5	2	5
Jβ 2.6	15	10

Table 2 – Comparison of the sequences between the end of Vβ 8.1 region and the beginning of the two more frequent Jβ sequences (Jβ 2.6 and Jβ 2.1) used for both C57BL/6 and JH-/- strains. A total of 20 (in the case of C57Bl/6) and 36 sequences (in the case of JH-/-) were studied. The NCBI BLAST software was used to find the Vβ 8.1 sequence and the Jβ sequences were taken from the literature [21, 188].

Clone	**End of Vβ 8.1**	**D+N+P**	**Start of Jβ 2.6**
	TGTGCCAGCAGTG		CCTATGAACAGT
JH #1	TGTGCCAGCAGTG	AAAGGACTGGG	GAACAGT
JH #2	TGTGCCAGCAGTG	AAGGGGGGT	CCTATGAACAGT
JH #3	TGTGCCAGCAGTG	CG	TATGAACAGT
JH #4	TGTGCCAGCAGTG	ATAGAAGCT	CCTATGAACAGT
JH #5	TGTGCCAGCAGTG	ATGCAGGGACTGGGG GGA	ATGAACAGT
JH #6	TGTGCCAGCAGTG	CG	TATGAACAGT
JH #7	TGTGCCAGCAGTG	ATGCACTGGGGG GGTT	TGAACAGT
JH #8	TGTGCCAGCAGTG	TCCGGGACAGCT	CCTATGAACAGT
JH #9	TGTGCCAGCAGTG	TCCACTGCA	CTATGAACAGT
JH #10	TGTGCCAGCAGTG	ACAGATCCTA	TATGAACAGT
Clone	**End of Vβ 8.1**	**D+N+P**	**Start of Jβ 2.1**
	TGTGCCAGCAGTG		*ACTATGCTGAGC*
JH #11	TGTGCCAGCAGTG	ATCGGGACTGGGGGG	ATGCTGAGC
JH #12	TGTGCCAGCAGTG	GGGGTTATA	ACTATGCTGAGC
JH #13	TGTGCCAGCAGTG	AGGACTGGG	ACTATGCTGAGC
JH #14	TGTGCCAGCAGT	TCAG	ACTATGCTGAGC
JH #15	TGTGCCAGCAGTG	ATGGGGGGTT	CTATGCTGAGC
JH #16	TGTGCCAGCAGTG	ATACCTGGGGGGCCCTCA	ACTATGCTGAGC
JH #17	TGTGCCAGCAG	CTGACTGGGGGA	ACTATGCTGAGC
JH #18	TGTGCCAGCAGTG	ATGTCTGGGGTA	ACTATGCTGAGC

Clone	End of Vβ 8.1	D+N+P	Start of Jβ 2.6
	TGTGCCAGCAGTG		CCTATGAACAGT
BL/6 #1	TGTGCCAGCAGTG	GGGACAGAT	CCTATGAACAGT
BL/6 #2	TGTGCCAGCAGTG	CCCCAGACTGGA	CTATGAACAGT
BL/6 #3	TGTGCCAGCAGTG	ATGGAGGCT	CCTATGAACAGT
BL/6 #4	TGTGCCAGCAGTG	ACTTCT	CCTATGAACAGT
BL/6 #5	TGTGCCAGCAGTG	ATGCCGGACACT	CCTATGAACAGT
BL/6 #6	TGTGCCAGCAGTG	AAG	CCTATGAACAGT
BL/6 #7	TGTGCCAGCAGTG	GGGGGGC	CCTATGAACAGT
BL/6 #8	TGTGCCAGCAGTG	ACTCCGGGACAGGGTACG	ATGAACAGT
BL/6 #9,10,11	TGTGCCAGCAGTG	CTGACTGGGGGGCCT	CCTATGAACAGT
BL/6 #12	TGTGCCAGCAGTG	GAGGGGGGGGGAT	CCTATGAACAGT
BL/6 #13	TGTGCCAGCAGTG	ATAGGCAGGGCT	ATGAACAGT
BL/6 #14, 15	TGTGCCAGCAGTG	AGCAGTGAAGGGAC	CCTATGAACAGT
Clone	**End of Vβ 8.1**	**D+N+P**	**Start of Jβ 2.1**
	TGTGCCAGCAGTG		*ACTATGCTGAGC*
BL/6 #16	TGTGCCAGCAGTG	CTGACTGGGGCCTTA	ACTATGCTGAGC
BL/6 #17	TGTGCCAGCAGTG	ATTACTGGGGGGAAAGG	TATGCTGAGC
BL/6 #18	TGTGCCAGCAGTG	CTCAGATT	CTATGCTGAGC
BL/6 #19	TGTGCCAGCAGTG	ATGCAAGGGGT	ACTATGCTGAGC
BL/6 #20	TGTGCCAGCAGTG	AGCCGGGAC	ACTATGCTGAGC
BL/6 #21	TGTGCCAGCAGTG	ATTACAGGGG	CTATGCTGAGC
BL/6 #22	TGTGCCAGCAGTG	ATGAGGGGA	ACTATGCTGAGC

5.4.3. Increased *in vivo* proliferation of T cells on B cell deficient mice

In B cell-deficient mice (JH-/-) and in mice with oligoclonal B cells (QM), contracted thymocyte repertoires [185] may contribute to the contracted

TCR Vβ diversity of splenocytes. Additional mechanisms, however, such as homeostatic proliferation in response to decreased numbers of T cells [189, 190] may also contribute to contracted peripheral T cell repertoire in B cell deficient mice. Homeostatic proliferation is thought to cause a contraction in the TCR repertoire because normalization of T cell numbers is owed to clonal expansion rather than to de novo production of T cells [189, 190].

JH-/- mice have reduced numbers of T cells in the spleen (CD3+ T cells are 33% of wild type, respectively) (Figure 2A) that could, in principle, trigger homeostatic proliferation. T cells that result from in vivo homeostatic proliferation acquire phenotypic markers of memory and are for this reason often referred to as "memory like". "Memory like" T cells express high levels of adhesion molecules such as CD44 reflecting specific requirements to enter peripheral tissues [191]. To determine whether in vivo peripheral proliferation occurs in these mice, we compared the relative numbers of "memory like" and naïve $CD4^+$ T cells in JH-/- and QM with those in wild type mice. Figure 3A shows that the proportion of "memory like" $CD4^+$ T cells (CD4+/CD44hi) is increased in JH-/- compared to the proportion of these cells found in QM and C57BL/6 mice (the mean proportion for JH-/- is 1.74 and 1.5 times more compared to the mean proportion of CD4+/CD44hi in the wild type and QM mice, respectively). These results do favor homeostatic proliferation secondary to a primary reduction in the numbers of peripheral T cells and suggest that the reduction of T cell diversity in JH-/- could be also

secondary to the compensatory peripheral oligloclonal expansion besides the effect of lacking B cells and Ig. In the QM mice, the contraction of the peripheral T cell repertoire may thus reflect accurately the contracted repertoire of new T cells and/or a defective maintenance of diverse T cell clones in B cell deficient mice or mice with oligoclonal B cells.

The expression of the Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen is scattered within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G(1), S, G(2), and mitosis), but is absent from resting cells (G(0)), makes it a sensitive marker for determining proliferative cells. Figure 3B shows Ki-67 staining of spleen sections of JH-/-, QM and C57BL/6 mice. In the case of JH-/- mice, the proliferative ratio given by the proportion of positive Ki-67 cells in a spleen section is significantly higher compared to what is seen in the QM or C57BL/6 spleens. To, more precisely, quantify the proportion of T cells that were proliferating, cell cycle analysis of isolated splenic T cells of age matched JH-/-, QM and C57BL/6 was performed. In JH-/- mice, the average proportion of splenic T cells in phase S+G2 was 1.76 and 2.25 times the averages in the wild type and QM mice, respectively. In one case, the proportion of T cells proliferating in the JH-/- mice was 5 times higher than the proportion of proliferating T cells in

C57BL/6 or QM mice. In QM mice, the proportion of proliferating T cells did not differ from the proportion of T cells in C57BL/6 mice (figure 3C).

Figure 3

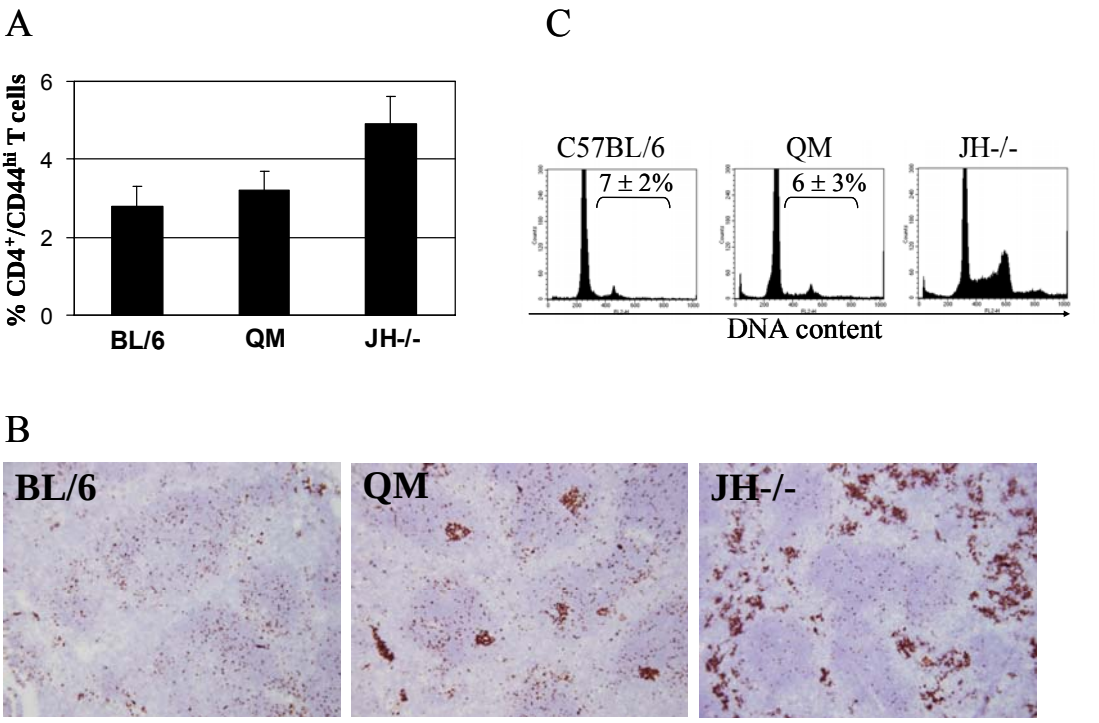


Figure 3 – Memory phenotype and *in vivo* proliferation studies in the spleen of B cell compromised mice compared to wild type. A – CD4+ T cell memory phenotype. *y-axis*, % of memory $CD4^+$ T cells in the spleen. *x-axis*, mice strains. Memory $CD4^+$ T cells were characterized as $CD4^+/CD44^{hi}$ by FACS analysis. In the graph each bar represents the mean and the error bars represent standard deviation of each distribution. In this analysis were used 4 C57Bl/6, 4 QM and 2 JH-/- adult, age-matched mice. Statistical comparison was performed using the Wilcoxon test. **B – Ki-67 staining of spleen sections.** B cell compromised mice were compared to wild type mice. **C – Cell cycle analysis.** DNA content was analyzed by flow cytometry in T cells sorted from spleens of 6 C57BL/6, 3 QM and 4 JH-/- age–matched mice. The numbers displayed correspond to the mean ± standard deviation of the percentage of T cells on phase S+G2 of cell cycle in

each mice strain. The DNA content charts displayed correspond to one example of each mice strain.

5.4.4. Delayed rejection of H-Y incompatible grafts by JH-/- and QM mice

T cell immunological competence is generally thought to be at least in part, a function of T cell repertoire diversity because T cell responses to most microorganisms and to minor histocompatibility antigens depend on rare precursors expressing antigen specific TCR [192-194]. To determine whether contraction of the T cell repertoire impacts T cell function, we studied T cell responses to skin grafts in mice, avoiding the impact of humoral rejection. Also testing rejection to minor histocompatibility antigens enable the detection of small differences in T cell function that may not be possible by testing responses to major histocompatiblity antigens, where rejection is usually too fast to allow detection of small variations on T cell function. Overall, 12 C57BL/6 and 5 JH-/- female mice were evaluated, where all had documented skin graft rejection. Ten QM female mice were also evaluated, where 7 of these mice had documented skin graft rejection and the remaining 3 were censored at their last evaluation timepoint. The median time to skin graft rejection for C57BL/6 female mice was 16 days. Figure 4 shows that JH-/- and QM female mice had a significant increase in

time to skin graft rejection, with medians of 22 days ($p = 0.0006$) and 43 days ($p = 0.00001$), respectively. These results support that TCR diversity is associated with T cell function and thus that a decrease in TCR diversity may cause a decrease in T cell function. However, TCR diversity alone may not determine the outcome of skin grafts, where JH-/- mice had a smaller TCR diversity than QM mice but rejected skin grafts significantly faster than QM mice ($p = 0.04$). These results suggest instead that T cells with a low level of diversity in the JH-/- stimulated to actively proliferate may acquire properties that allow them to reject H-Y incompatible skin grafts earlier than QM T cells that have a higher TCR diversity but are not so actively proliferating.

Figure 4

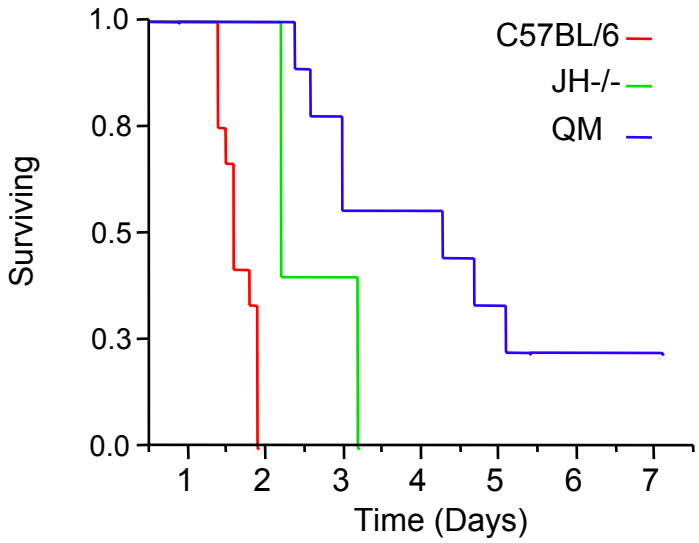


Figure 4 - Kaplan Meier survival curves for H-Y incompatible skin grafts in C57BL/6, QM and JH-/- mice. x-axis, days following surgery; y-axis, skin graft survival fraction. Rejection was defined by shedding 90% or more of the graft from the graft bed. The median time of rejection was 16 days in C57BL/6; 43 days in QM and 22 days in JH-/- mice. QM and JH-/- skin grafts survived significantly longer compared to C57BL/6, $p < 0.0001$ and $p = 0.0006$, respectively. Each mice group included 12 C57BL/6, 10 QM and 5 JH-/-. All mice were grafted 5 to 6 weeks after birth. Statistical analysis was done with the Log-rank test.

5.4.5. Exclusion of other immunological factors that, besides contracted TCR diversity, may decrease T cell function:

1. Activation potential of T cells of B cell compromised mice.

To test whether the intrinsic ability of T cells to respond to activation was unaltered in JH-/- or QM mice compared to wild type mice we tested in vitro proliferation of CD4+ T cells in response to polyclonal stimuli, anti-CD3 and anti-CD28. A lack of responsiveness by the T cells could be, in fact, a reason for the delayed rejection of the skin transplants. Figure 5A shows that CD4+ T cells obtained from QM and JH-/- mice appear to proliferate as well as cells from wild type mice following stimulation. These results indicate that the T cells of JH-/- or QM mice retain wild type primary responsiveness to polyclonal stimuli and that the delayed function of T cells in rejection the H-Y incompatible skin grafts is not related to lack of ability for these cells to respond. Also, these data can be described as a trend to a higher proliferation response of the CD4+ T cells to polyclonal stimuli, although statistically not significant, when comparing the CD4+T cells response from mice that lack B cells to the CD4+T cells response of strains of mice that are B cell proficient.

2. Antigen presentation ability of quasi-monoclonal B cells.

Delayed rejection of H-Y incompatible skin grafts could also be due to a deficiency of antigen presentation by B cells. Since JH-/- mice lack B cells, we tested this possibility by comparing antigen presenting properties of QM and wild type B cells. To this end, we assessed proliferation of DO11.10 T

cells in the presence of specific antigen (the OVA peptide 323-339) in the presence of wild type or quasi-monoclonal B cells. Figure 5B shows that proliferation of DO 11.10 T cells is comparable whether the OVA peptide is presented by wild type or by QM B cells. Our results indicate that antigen presentation by the QM B cells is not compromised.

3. Proportion of CD4+/CD25+ T cells in B cell compromised mice compared to wild type.

The presence of T cells with suppressor properties (CD4+CD25+ T cell phenotype) are thought to promote survival of allografts [195]. To exclude the possibility that the longer survival of H-Y incompatible skin grafts in QM and JH-/- mice with contracted T cell repertoires was accompanied by an increased percentage of CD4+CD25+ T cells, we assessed the proportion of these cells in these mice strains compared to wild type. Figure 5C shows that the frequencies of CD4+CD25+ T cells in the peripheral blood are similar in JH-/- mice and QM mice compared to wild type mice, suggesting that suppression owing to T regulatory cells may not contribute to the increased survival of H-Y incompatible skin grafts in JH-/- and QM mice.

Figure 5

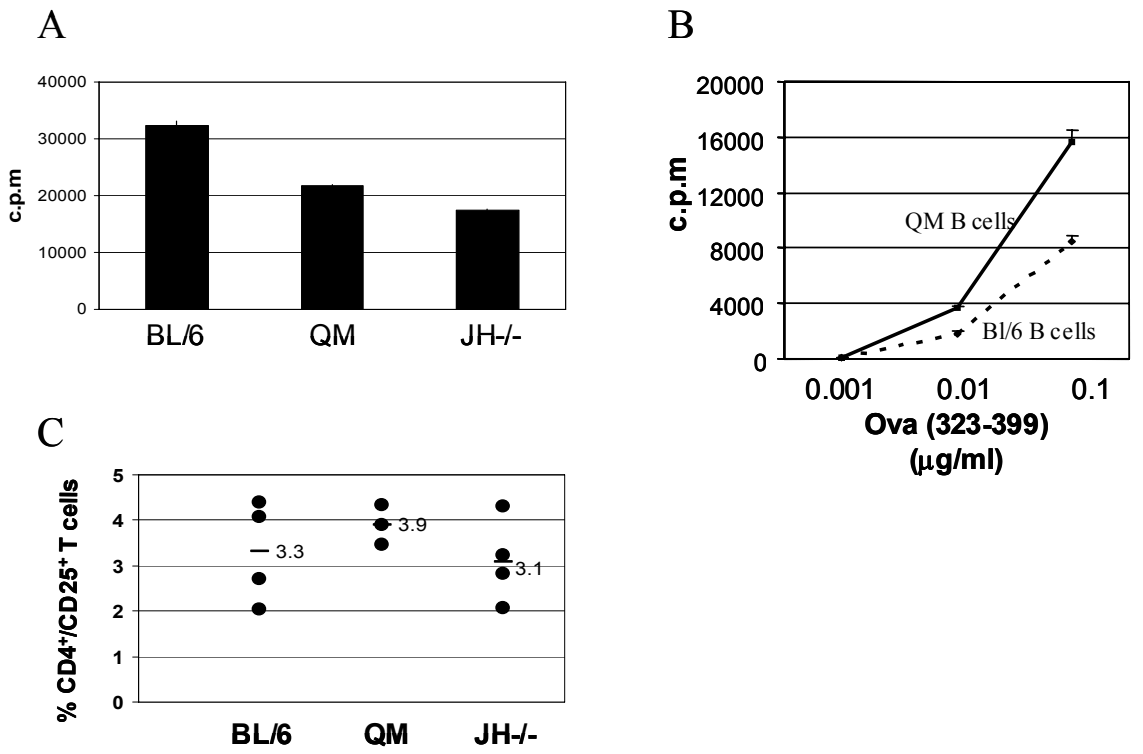


Figure 5. A) *In vitro* proliferation ability of CD4+T cells against polyclonal stimuli in C57BL/6, QM and JH-/- mice. *x-axis*, mice strains; *y-axis*, counts per minute. Purified $CD4^+$ T cells from the different strains of mice were cultured for 4 days with 10 μg/ml (coated well) and 10 μg/ml of anti CD28 and proliferation was assessed by the incorporation of [^{3}H]thymidine (1 μCi/well) during the last 16 h of culture. In these experiments, background counts in the wells containing just cells or just media were all < 250 cpm. Results are expressed as the mean ± SEM of triplicate samples of one example from 4 independent experiments in the case of C57BL/6 and QM mice and 2 independent experiments for the JH-/- mice. There is no statistically difference between the groups (p>0.05,

Student's *t* test). **B) *In vitro* proliferation response of DO 11.10 T cells to OVA (323-339) presented by wild type or QM B cells.** 2 x 10^4 purified $CD4^+$ T cells specific to OVA 323-339 were cultured for 4 days in the presence of 2 x 10^5 irradiated B cells taken from C57BL/6 (dotted line) or QM mice (continuous line) with three crescent concentration of soluble OVA 323-339 peptide. Proliferation was assessed by the incorporation of [^{3}H]thymidine (1 μCi/well) during the last 16 h of culture. In these experiments, background counts in the wells containing just cells or just media were all < 150 cpm. Results are expressed as the mean ± SEM of triplicate samples.

C) Fraction of $CD4^+/CD25^+$ splenocytes measured by flow cytometry analysis. *x-axis*, % of $CD4^+/CD25^+$ splenocytes ; *y-axis*, mice strains. Each circle represents one experiment, and the values indicate the mean % of the $CD4^+/CD25^+$ population for each strain of mice. There is no statistically difference between the groups ($p>0.05$, Student's *t* test).

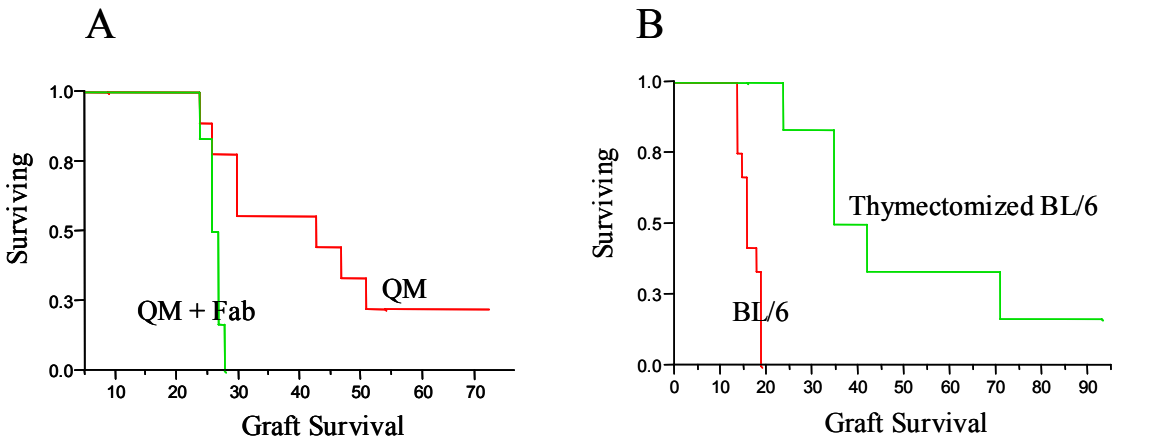
5.4.6. Recovery of TCR diversity and T cell function

Our data suggest that B cell-dependent T cell repertoire diversity is a major factor determining the kinetics of rejection of grafts across minor antigen barriers. If the magnitude of the T cell repertoire predicts immune responses across minor antigen barriers, then increasing diversity in mice with a contracted T cell repertoire and normal lymph node architecture should hasten rejection of H-Y incompatible skin grafts. To test this prediction we increased T cell diversity of QM mice by administering immunoglobulin Fab

fragments or polyclonal IgG. We previously showed that injecting polyclonal Ig on mice with contacted TCR repertoire increase T cell diversity [185]. However, treatment with Ig molecules is also known to generate immunologic suppression mainly because of the attributes of the Fc fraction of the molecule [196]. We tested if the injection of Ig Fab fragments would increase TCR diversity without generating immune suppression, but, on the contrary, improving T cell function. Figure 6A displays how Fab-injected QM mice (n=3) recovered TCR Vβ diversity to wild type mean levels (13.4 x 10^5 compared to 6.0 x 10^5 in C57BL/6). The QM mice treated with Ig Fab fragment (n=6) rejected H-Y incompatible skin grafts significantly faster than non injected QM mice (n=10) (medians: 27 vs. 43 days, respectively; p=0.007). The QM mice treated with polyclonal IgG (n=4) also rejected H-Y incompatible skin grafts faster than not treated QM mice (n=10), although not achieving statistically significance, p= 0.07. In a complementary approach, we tested whether decreasing TCR repertoire in the wild mice prolonged survival of H-Y incompatible skin allografts. We performed thymectomies in 5 week-old C57BL/6 mice (n = 8) and measured TCR Vβ diversity 6 weeks after the surgery. Figure 6B shows that thymectomized mice have a substantial contraction of TCR repertoire compared with non-thymectomized C57BL/6 mice (n=3), (means: 60.4 x 10^4 in non-thymectomized C57BL/6 vs. 1.5 x 10^4 in thymectomized C57BL/6). In addition, thymectomized females transplanted with male skin 9 weeks after thymectomy (n = 7) allowed survival of the grafts significantly longer than

non-thymectomized C57BL/6 females (n=12), with median time to skin graft failure of 38.5 days vs. 16 days, respectively (p = 0.0001). This suggests that decreasing TCR diversity leads directly to decline of T cell function.

Figure 6



	TCR Vβ diversity
QM	$7.7 \times 10^3 \pm 1.2 \times 10^4$
QM + Fab	$1.3 \times 10^6 \pm 2.2 \times 10^6$

	TCR Vβ diversity
BL/6	$6.0 \times 10^5 \pm 3.3 \times 10^5$
Thymectomized BL/6	$1.5 \times 10^4 \pm 3.4 \times 10^4$

Figure 6. Survival of H-Y incompatible skin grafts in QM mice where TCR Vb diversity was reconstituted compared to non-reconstituted QM mice (A) and in wild type mice (C57BL/6) where TCR Vβ diversity was decreased by thymectomy (B) compared to wild type.

A) TCR Vβ diversity in QM mice injected with Fab fragment (n=3) compared to non injected QM mice (n=4). There is, in mean, an increase of 174 times in the TCR Vβ diversity after treatment with Ig Fab fragment, what achieved, in mean, wild type TCR Vβ diversity values. The grafts survival study show that QM mice treated with Ig

B) Fab (n=6) have a faster rejection of H-Y incompatible skin grafts compared to non treated QM mice (n=10), p= 0.007, Log rank test. There was also a faster rejection of H-Y incompatible skin grafts in QM mice injected with polyclonal Ig (n=4) compared to not injected QM mice (n=10), although not achieving statistically significance, p= 0.07, Log rank test. **B)** TCR Vβ diversity in thymecthomized wild type mice (n=8) compared to non thymectomized wild type mice (n=3). There is, in mean, a decrease of 40 times in the TCR Vβ diversity measured 6 weeks after thymectomy. The grafts survival study show that thymecthomized wild type mice (n=7) have a slower rejection of H-Y incompatible skin grafts compared to non-thymecthomized wild type mice (n=12), p= 0.0001, Log rank test.

5.5. Discussion

The data presented here demonstrate that B cells and/or immunoglobulin are a major determinant of diversity and, consequently, of the function of peripheral T cells and that immunoglobulin derivatives can be used to improve T cell function.

The effect of Ig promoting TCR diversification previously reported by us [185] might be related with the highly diversity of the Fab fragments presented, independently of the Fc portion of the molecule. It is known that the repertoire diversity is dependent of the diversity of the antigens presented in the thymus [149, 151]. Thus, it would be possible to maintain the positive effect on TCR diversity and function without the immunosuppressive activity of the Fc portion of the molecule, by using Fab fragments instead of the whole Ig molecule. Here we show that *in vivo* administration of immunoglobulin Fab fragments allowed the reconstitution of a T cell repertoire similar to the mean wild type diversity, and avoided the immunological suppressive effects of polyclonal immunoglobulin.

In an additional approach we demonstrated that the decrease on TCR Vb diversity achieved by surgical removing of the entire thymus led to a decrease on T cell function, shown by the significant delayed rejection of skin grafts. This is in agreement with previous studies reported that patients who suffered thymus removal consequently achieved a worse function of the T

cell compartment [197, 198]. Thymectomies may affect other T functions besides decreasing TCR repertoire. For example, thymectomy may decrease T cells suppressor functions and may stimulate homeostatic proliferation and memory like activity of T cells. However, the effect of decreasing T cell suppression or of promoting homeostatic proliferation with increase of memory properties would be expected to have the opposite effect on the skin allografts rejection compared to the effect of decreasing TCR diversity. Thus, our results seem to indicate that the decrease on TCR diversity caused by thymectomy is dominant over other possible consequences of thymectomy, as the ones stated above.

The effect of TCR diversity was proved independent of other factors that entail rejection of skin grafts namely T cell ability to respond to antigens, antigen presentation, organogenesis of peripheral lymphoid organs and suppressor activity of regulatory T cells.

Taken together this data demonstrate a new compass of the T-cell -B-cell bidirectional cooperation: that B cells and immunoglobulin are fundamental in the generation and maintenance of a diverse compartment of T cells, affecting T cell function *via* that mechanism. Idiotype peptides of B cell immunoglobulin may be the driving force for the antigen presenting function of B cells to influence the T cell repertoire. This would be another relevance of the Jerne's idiotypic network.

We suggest that B cell primary immunodeficiencies such as Bruton's syndrome are, in some degree, common B-cell/T-cell immunodeficiencies.

Evidence in experimental animals and in man indicated previously that immunoglobulin may select pre-immune B cell repertoires in the bone-marrow and peripheral lymphoid tissues [199, 200]. We previously showed that B cells and Ig contributed to the generation of thymocyte TCR diversification [185]. The role of Ig as a self molecule used by the body to select B and T repertoire is a changing and new model idea that emerges from these studies and deserves extended mechanistic and translational studies. One mechanistic hypothesis put forward by our studies is that B cells acting as antigen presenting cells of secreted or endogenous Ig-derived peptides could present an extensive diversity of peptides to T cells precursors, generating a wider diverse repertoire of newly formed T cells.
In the periphery, it is known that newly formed T cells require a constant engagement of the TCR with self-ligands to persist in a quiescent state [201, 202]. B cells, by means of Ig, may continuously allow T cell "tickling" by self-peptide/MHC complexes. This mechanism would warrant survival of naïve T cells and maintenance of a diverse repertoire of T cells.

The fundamental role of B cells and Ig is manifest on naïve T cells. By the contrary, memory T cell function is not notable dependent on B and other antigen presenting cells. Once memory is generated, T cells survive and proliferate without antigen [203] and memory T cells persist in MHC class I [204] and II [205] knockout. The effect of decrease T cell repertoire on memory responses was not the objective of this study, which only examined

the effect of TCR diversity on primary T cell function, given by the rejection of first H-Y incompatible skin grafts.

This study also shows that in situations where homeostatic proliferation is present as a compensatory mechanism for the decrease on the number of T cells, the cells acquire properties possibly counterbalancing the deficit in T cell function achieved by the contraction of repertoire diversity. However, to which level this compensatory mechanism exert its effect, how differently this effect occurs in naïve and memory T cell populations and how can it be optimized and exploited is not yet known.

Futures studies on the mechanism of how Ig peptides promote TCR diversity, on the specific role of each fragment of the Ig molecule and on the interaction between Ig and TCR molecules should be undertaken with the purpose of clearly understanding how Ig derivatives promote T cell function by increasing TCR diversity. This fact brings the possibility of improving immunity for a vast number of patient populations undergoing immune reconstitution. We believe that our results open a new therapeutic perspective in situations where immune reconstitution occurs. This may have profound implications for the adjuvant treatment of conditions associated with immune deficiency such as AIDS, autoimmunity, cancer, post myeloablative therapy.

CHAPTER VI

Early lymphocyte recovery after autologous stem cell transplantation predicts superior survival in Mantle Cell Lymphoma

Early lymphocyte recovery after autologous stem cell transplantation predicts superior survival in Mantle Cell Lymphoma

Cristina João MD[1 2], Luis F. Porrata MD[2], David J. Inwards MD[2], Stephen M. Ansell MD, PhD[2], Ivana N. Micallef MD[2], Patrick B. Johnston MD, PhD[2], Dennis A. Gastineau MD[2], Svetomir N. Markovic MD, PhD[2]

[1] Hematology Department, Portuguese Institute of Oncology, Lisbon, Portugal
[2] Division of Hematology/Department of Internal Medicine, Mayo Clinic College of Medicine, Rochester MN, 55905

This study was funded by the University of Iowa, Mayo Clinic Lymphoma SPORE CA97274, USA and Fundação para a Ciência e Tecnologia, Lisbon, Portugal (BD 6797/2001).

Bone Marrow Transplantation 2006, 37:865-871

6.1. Abstract

Autologous stem cell transplantation (ASCT) is an effective treatment strategy for mantle-cell lymphoma (MCL) demonstrating significantly prolongs progression free survival (PFS) when compared to IFNα maintenance therapy of patients in first remission. The study of ALC-15 after ASCT as a prognosis factor in NHL included different subtypes of lymphomas. The relationship of ALC-15 after ASCT in MCL has not been specifically addressed. Herein we present data evaluating the impact of ALC-15 recovery on survival of patients undergoing ASCT for MCL. We studied 42 consecutive MCL patients who underwent ASCT at the Mayo Clinic in Rochester from 1993 to 2005. Based on our previous studies, the ALC-15 threshold was set at 500 cells/μL. The median follow-up after ASCT was 25 months (range, 2-106 months). The median overall survival (OS) and PFS times were significantly better for the 24 patients that achieved an ALC-15 $\geq$ 500 cells/μL compared with 18 patients with ALC-15 < 500 cells/μL (not reached versus 30 months, $P < 0.01$ and not reached versus 16 months, $P < 0.0006$, respectively). Multivariate analysis demonstrated ALC-15 to be an independent prognostic factor for OS and PFS. ALC-15 $\geq$ 500 cells/μL is associated with significantly improved clinical outcomes following ASCT in MCL.

Keywords: absolute lymphocyte count, mantle cell lymphoma, autologous stem cell transplantation.

6.2. Introduction

Mantle-cell lymphoma (MCL) is characterized by an aggressive clinical course and poor prognosis with a median survival of only 3 to 4 years [206, 207]. Unlike other aggressive NHL, patients with MCL have a poorer complete response rate with standard anthracycline-based or non- anthracycline-based regimens [208, 209].

High-dose chemotherapy (HDC) followed by autologous stem cell transplantation is an effective form of treatment for relapsed non-Hodgkin's lymphoma [29]. In the case of MCL, this type of treatment has been shown relatively success, with 50 to 70% of patients free of disease at 2 or 3 years, if patients are transplanted in 1^{st} remission [210, 211]. The European MCL Network completed the only current available prospective randomized study comparing ASCT with IFNα maintenance in 1^{st} remission in patients with advanced stage MCL [212]. This multicenter trial demonstrates a significant improvement of PFS of patients with advanced stage MCL who were treated with myeloablative radiochemotherapy followed by ASCT compared to patients on IFNα maintenance. The outcome of patients with MCL after ASCT has been associated with age and levels of C-reactive protein at diagnosis, levels of B2-microglobulin at the time of diagnosis or transplantation, conditioning regimens including TBI, morphologic variants, tumor score and expression of p53 [213-215].

The absolute lymphocyte count (ALC) recovery ≥ 500 cells/μL at day 15 (ALC-15) after ASCT has been reported as a powerful and independent prognostic factor for clinical outcomes for patients with NHL [82, 216, 217], Hodgkin lymphoma [84, 216], multiple myeloma [82], acute myelogenous leukemia [218], amyloidosis [83], breast cancer [86, 219]. The fact that the recover of lymphocytes after ASCT affects survival of cancer patients is a clinical evidence of the existence of an autologous graft-versus-tumor effect.

To assess whether absolute lymphocyte count at day 15 post ASCT has prognostic significance in MCL post ASCT, we conducted a retrospective analysis of consecutive MCL patients undergoing ASCT since 1993 and 2005 and assessed OS and PFS.

6.3. Patients and Methods

4.3.1. Patient population. Forty two consecutive patients with the diagnosis of MCL underwent ASCT at the Mayo Clinic between November 1993 and March 2005. Data from transplant recipients used on this retrospective study were collected prospectively and entered into a computerized database. Response to therapy, relapse, and survival data are updated continuously. No patients were lost to follow-up. All patients gave written, informed consent allowing the use of their medical records for medical research. Approval for the retrospective review of these records was

obtained from the Mayo Clinic Institutional Review Board and was in accordance with US federal regulations and the Declaration of Helsinki.

6.3.2. End Points. The primary end point of the study was to determine if ALC -15 after ACST is a prognostic factor for PFS or (OS) in patients with MCL who underwent ASCT. The ALC-15 was calculated from the standard complete blood cell count at day 15 after ASCT.

6.3.3. Prognostic factors. This study used the following prognostic factors for patients with MCL: international age-adjusted prognostic index (IPI) [220] (age (≥60 vs <60), lactate dehydrogenase (LDH) > normal, performance status (PS-ECOG) (≥2 vs <2), extranodal sites (≥2 vs <2) and stage (III/IV vs I/II)), in addition to the number of pre-transplant treatments, stem cell source (BM vs PBSCs), type of conditioning regimen and disease status (CR1 / PR1 vs CR>1 / PR>1) before transplantation. The ALC-15 was determined at day 15 after ASCT.

6.3.4. Conditioning regimens. The conditioning regimens for patients were as follows: 9 patients received BEAC (BCNU [300 mg/m^2], Etoposide [100 mg/m^2], ARA-C [100 mg/m^2], and Cyclophosphamide [35 mg/kg]), 30

patients received BEAM (BCNU [300 mg/ m^2], Etoposide [100 mg/ m^2], ARA-C [100 mg/m^2], and Melphalan [140 mg/ m^2] and 3 patients received Cyclophosphamide [60 mg/ m^2] and total body irradiation [12 Gy]. All patients underwent stem cell re-infusion after HDC. Hematologic engraftment was thought to have occurred when the ANC reached 500 cells/μL or more for more than 3 consecutive days.

6.3.5. Stem cell source. The stem cell source for the ASCT included bone marrow (BM) or peripheral blood stem cells (PBSCs). 41 patients received PBSCs, and 1 received a mixture of PBSCs and BM stem cells. The decision to change from BM stem cell to PBSC was made when PBSCs became the standard for stem cell collection. The patient who did not obtain an adequate number of stem cells through PBSCs underwent BM harvest.

6.3.6. Response and survival. Complete remission (CR) was defined as complete regression of all measurable or evaluable disease including radiologically demonstrable disease, BM involvement, or peripheral blood involvement. Partial remission (PR) was defined as a reduction in the sum of the products of measurable lesions' longest diameters and perpendicular diameters of 50% or greater, with a 30% or greater decrease in hepatomegaly or splenomegaly (measured from the costal margin), if there

was previous known liver or spleen involvement. Disease progression was defined as a 25% or more increase in the sum of the products of the longest diameter and its perpendicular diameter of measurable lesion(s) from the pre-study measurement, the appearance of new lesions, or a 2-cm increase in spleen or liver size due to lymphoma.

OS time was measured from the date of transplantation to the date of death or last follow-up. PFS was defined as time from transplantation to disease progression, relapse, or death.

6.3.7. Statistical analysis. OS and PFS times were analyzed using the method described by Kaplan and Meier [221]. Differences between survival curves were tested for statistical significance using the 2-tailed log-rank test. The Cox proportional hazards model [222] was used to assess ALC-15 as a prognostic factor for post-transplant OS and PFS times as well as to adjust for other known prognostic factors. Hazard ratios reported are for risks associated with patients having high (greater than 500 cells/μL) versus low (less than 500 cells/μL) ALC values. The cutoff of an ALC of 500 cells/μL or more was based on data from our previous study [82]. Multivariate analysis done using Cox regression models tested all variables with a $P < 0.2$ in the univariated analysis.

Pearson's chi-square analysis was used to determine relations between nominal variables; nonparametric tests were used for continuous variables. All p values represented were 2-sided, and statistical significance was declared at $p < .05$.

The accuracy of the absolute number of lymphocytes recovered at day 15 after ASCT as a predictor of PFS during the study follow-up was evaluated by the Harrell's method, i.e., concordance (c)-statistic [223], what is equivalent to the area under the receiver operating characteristic (ROC) curve. This statistic may vary from 0 to 1, with 1 indicating perfect discrimination and 0.5 indicating what is expected by chance alone.

6.4. Results

6.4.1. Patient characteristics

A total of 42 patients (9 females and 33 males) were included in the study; the median age for the cohort group at transplant was 57 years (range, 37-71). Forty of the patients had a histologic type of MCL of intermediate variant and 2 had blastic mantle cell type. The analysis of the patients baseline characteristics that may impact survival and recovery of absolute lymphocytes counts after ASCT show similar distribution of those characteristics when comparing the patients who recovered the absolute count of lymphocyte at day 15 after ASCT (ALC-15 ≥ 500 cells/μL) and the patients who did not (ALC-15 < 500 cells/μL) (Table 1).

Table 1 – Baseline characteristics of patients according to the recovery of absolute lymphocyte count at day 15 after ASCT (ALC-15).

Characteristics	**ALC ≥ 500 cells/μL N = 24**	**ALC < 500 cells/μL N = 18**	**p value**
Age			p=0.92
≥ 60	7	5	
< 60	17	13	
Sex			p=0.51
Females	6	3	
Males	18	15	
LDH			p=0.76
≤ Normal	12	9	
> Normal	3	3	
PS (ECOG)			p=0.38
≥ 2	1	0	
< 2	23	18	
Stage (Ann Arbor)			p=0.16
I/II	5	1	
III/IV	19	17	
Number of pre-transplants treatments			p=0.90
1	12	10	
2	11	7	
3	1	1	
Conditioning Regimen			p=0.23
BEAC	2	4	
BEAM	20	14	
Cyclophosphamide/TBI	2	0	
Disease Status at Transplant			p=0.18
CR1 or PR1	13	6	
>CR1 or >PR1	11	12	
# $CD34^+$ cells transplanted			p=0.12
Median (range)	4.37 x 10^6 (2.28-8.44 x10^6)	2.87 x 10^6 (2.0-10.3 x10^6)	
Post-transplant cytokines			p=0.40
G-CSF	3	4	
GM-CSF	21	14	

Abbreviations: ASCT = autologous stem cell transplantation; BEAC = BCNU (300mg/m^2), Etoposide (100mg/m^2), ARA-C (100mg/m^2), and Cyclophosphamide (35mg/Kg); BEAM = BCNU (300mg/m^2), Etoposide (100mg/m^2), ARA-C (100mg/m^2), and Melphalan (140mg/m^2); CR1 = complete response 1; PR1 = partial response 1; LDH = lactate dehydrogenase; PS = performance

status; 2-CDA = cladribine (3,4mg/m^2); CHOP = Cyclophosphamide (750 mg/m^2), Doxorubicin (50mg/m^2), Vincristine (1,4mg/m^2), and Prednisone (100mg/m^2); R-CHOP = Rituxan (375mg/m^2), Cyclophosphamide (750 mg/m^2), Doxorubicin (50mg/m^2), Vincristine (1,4mg/m^2), and Prednisone (100mg/m^2); HperCVAD = Cyclophosphamide (300mg/m^2), Doxorubicin (50mg/m^2), Vincristine (2mg/day), and Dexametasone (40mg/day).

6.4.2. Prognostic factors for progression and survival

The median follow-up after ASCT was 25 months (range, 2-106 months). At the time of analysis, 25 out of the 42 patients were alive and 17 were dead.

The median survival was 47 months. The causes of death were AML in 1 case, progression of lymphoma in 14 patients, sepsis in 1 case and not known in 1 case. The patient who died with AML had ALC-15 ≥ 500 cell/μL; from the 14 patients who died of lymphoma progression only 3 had ALC-15 ≥ 500 cell/μL and the patient who died in sepsis had ALC-15 < 500 cell/μL. All the patients were treated similarly regarding supporting care (antibiotics, transfusions, growth factors) according to Mayo Clinic protocols.

The median OS (figure 1) and PFS (figure 2) times were significantly better for patients with an ALC-15 ≥500 cell/μL than for those with an ALC-15 < 500 cell/μL (not reached versus 30 months, $p < 0.01$ and not reached versus 16 months, $p < 0.0006$, respectively).

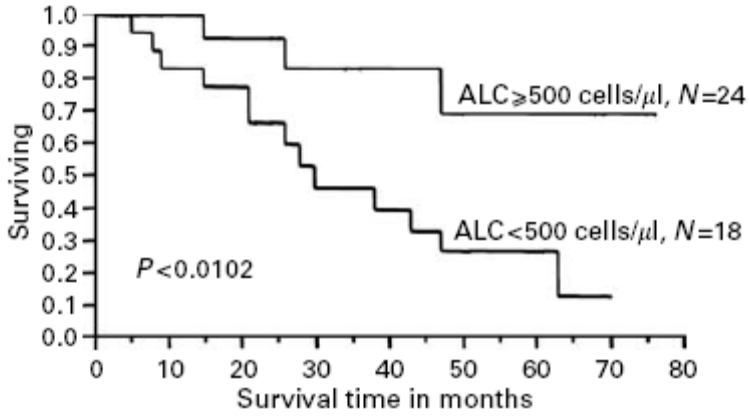


Figure 1 – Overall survival for 42 patients with mantle cell lymphoma after ASCT as a function of recovery of absolute number of lymphocytes at day 15. Median overall survival for patients with ALC ≥ 500 cells/μL was not reached and median overall survival for patients with ALC < 500 cells/μL was 30 months (p=0.01, log rank test).

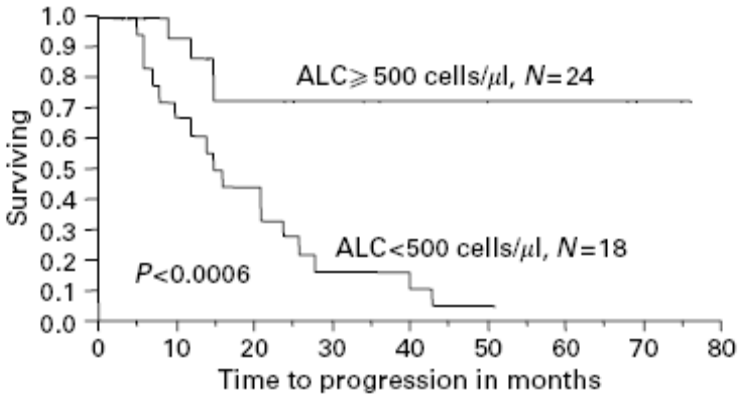


Figure 2 – Progression free survival for 42 patients with mantle cell lymphoma after ASCT as a function of recovery of absolute number of lymphocytes at day 15. Median overall survival for patients with ALC ≥ 500 cells/μL was not reached and median overall survival for patients with ALC < 500 cells/μL was 16 months (p=0.0006, log rank test).

Prognostic factors were tested as predictors for OS and PFS for the 42 patients with mantle cell lymphoma studied (Table 2). Age at transplant (≥ 60 vs < 60), LDH, stage (III/IV vs I/II), IPI ≥ 2 versus < 2, number of pre-transplant chemotherapy regimens (1 vs 2 or 3) and type of condition regimen were not predictor of OS or PFS. Recover of absolute lymphocyte count at day 15 after ASCT (≥ 500 cells/μL vs < 500 cells/μL) and being transplanted on first CR / first PR versus second or more CR/PR were significant predictors of OS and/or PFS on univariate analysis. Age at transplant was also tested as a continuous variable and it was not associated

to disease progression or patient survival (data not shown). The effect of condition regimens were tested comparing patients conditioned with BEAC to patients conditioned with BEAM. The 3 patients who did Cyclophosphamide/TBI were excluded. Presence of disease at extranodal sites and performance status (PS) were not included in the analysis because only 1 patient had 2 or more extranodal sites involved and only one patient had a PS ≥ 2.

Table 2 – Univariate analysis for patients with mantle cell lymphoma – overall survival and progression free survival rates were indicated.

Prognostic factors at time of transplantation	***Overall survival***			***Progression free survival***		
	HR	***95% CI***	***p value***	***HR***	***95% CI***	***p value***
Age (≥ 60 vs < 60)	0.84	(0.23 – 2.43)	0.77	1.06	(0.38 - 2.62)	0.90
LDH (normal vs > normal)	0.51	(0.03 – 2.89)	0.49	0.73	(0.11 – 2.81)	0.67
IPI (≥ 2 vs < 2)	1.08	(0.37 – 2.9)	0.87	1.48	(0.60 – 3.51)	0.38
Stage (III/IV vs I/II)	1.64	(0.31 – 30)	0.61	3.85	(0.79 – 69)	0.11
Number of pre-ASCT chemotherapy regimens (1 vs 2 or 3)	0.89	(0.31 – 2.45)	0.83	0.75	(0.31 – 1.80)	0.51
Condition regimen (BEAC vs BEAM)	1.35	(0.78 – 2.32)	0.27	1.34	(0.83 – 2.11)	0.21
ALC (≥ 500 cells/μL vs < 500 cells/μL)	**0.22**	**(0.05 – 0.70)**	**0.0086**	**0.19**	**(0.05–0.49)**	**0.0005**
Disease status at ASCT (CR1/PR1 vs CR>1/PR>1)	0.42	(0.11 – 1.21)	0.11	**0.25**	**(0.08–0.65)**	**0.0037**

HR – Hazard ratio;
95% CI – 95% Confident interval.

Having a recovery of the absolute number of the lymphocytes ≥500 cells/μL at day 15 after ASCT is an independent prognostic factor for OS (HR = 0.25, p = 0.02) and PFS (HR = 0.18, p = 0.0006) in this group of patients with MCL in multivariate analysis adjusted to disease status at transplant (Table 3). The hazard risk for death is 4 times less for patients who recover the number of lymphocytes to values ≥500 cell/μL by day 15 after ASCT compared with MCL patients who do not recover this value of ALC-15.

When the risk of lymphoma recurrence was assessed based on ALC-15, the hazard risk of recurrence was 5 times less for patients who recover ALC-15 ≥ 500 cell/μL.

Table 3 – Multivariate analysis for patients with mantle cell lymphoma – overall survival and progression free survival rates were indicated. Multivariate model contains all the prognostic factors listed.

Prognostic factors at time of transplantation	***Overall survival***			***Progression free survival***		
	HR	***95% CI***	***p value***	***HR***	***95% CI***	***p value***
ALC (≥ 500 cells/μL vs < 500 cells/μL)	**0.24**	**(0.06 – 0.80)**	**0.02**	**0.18**	**(0.05 – 0.50)**	**0.0006**
Disease status at ASCT (CR1/PR1 vs CR>1/PR>1)	0.54	(0.15 – 1.58)	0.27	**0.25**	**(0.08 – 0.66)**	**0.005**

HR – Hazard ratio;
95% CI – 95% Confidence interval.

Also, the multivariate analysis demonstrates that the disease status, i.e., being in first CR o/ first PR, is an independent prognostic factor for lymphoma recurrence, independent of the immune recovery after ASCT in terms of ALC-15. Patients who were transplanted in CR1 or PR1 have a 4 times less risk of recurrence compared to patients who were transplanted in second or more CR or PR (HR =0.25, p = 0.005).

6.4.3. Discrimination of ALC-15 in MCL patients as predictor of PFS

To compare the accuracy of ALC-15 as predictor of recurrence accounting for censored data, the Harrell's method, which yields the concordance c statistic, was calculated [223]. The c index estimates the probability that, of two randomly chosen patients, the patient with the higher value of the prognostic factor will outlive the patients with the lower value. Values of c near 1 indicate that a higher value of the prognostic factor virtually always determine that a patient has a better prognosis.

This new independent prognosis factor (ALC-15) alone can predict recurrence of MCL after ASCT with a discrimination power of 61%.

6.5. Discussion

Our study shows that recovery of the absolute number of lymphocytes after ASCT is a strong and independent prognostic factor for overall survival and progression free survival in patients with MCL who are treated with high dose chemotherapy followed by ASCT. Patients with MCL who recover the number of lymphocytes to values ≥500 cells/μL by day 15 after ASCT have a 5 times less risk of progression and a 4 times less risk of not surviving compared to patients with an ALC < 500 cells/μL by day 15 after ASCT.

A ROC curve provides a graphical representation of the relationship between the true-positive and false-positive prediction rate of a test and allows physicians and investigators to determine which should be the cut point value of the prognostic factors to use in order to best discriminate the expected outcome. The c-statistic allows the quantification of the discrimination power of prognostic factors of time-dependent events. The quantitative-qualitative relationship between the c-statistic and discrimination power of the prognostic factor follows a fairly linear pattern.

The value of 61% represents that the accuracy of ALC-15 predicting recurrence of MCL after ASCT in this group of patients is satisfactory. The knowledge of how accurate is a prognostic factor predicting the outcome helps physicians to rely on the prognosis factors when proposing treatment to their patients. Also, allow developing of alternative treatment strategies

to groups of patients predicted to early recurrence or death. From our data, it is clear that patients with MCL who do not recover the lymphocyte number by day 15 after ASCT should be offer new and investigative therapeutic strategies, as the risk of early recurrence or death is very high.

This is the first time these results are published for patients with MCL subtype of NHL. Before, we had shown the association between ALC-15 and overall survival and progression free survival in NHL [82], which included several histological subtypes. In the case of more aggressive NHL, as it is the case of MCL, the importance of being aware of prognostic factors that could be therapeutically modified and that very clearly divide the group of transplanted patients in those who have a bigger overall survival and PFS or not, is a key factor to better treat patients. Our study also shows that ASCT does not offer cure to MCL patients as the overall survival curve does not reach a plateau if patients are analyzed independent of theirs lymphocyte recovery. Immunotherapy comes as a reasonable treatment option on these patients after ASCT as other investigators have been investigating [224, 225]. However, the benefit of immunotherapy may be investigated in the context of the patients own immune system recovery and potential. One hypothesis is that the maximal effect of immunotherapy, including monoclonal antibodies or cytokine treatment, depends on the patient's own immune system robustness at the time of treatment.

The multivariate analysis indicates that the ALC-15 ≥500 cells/mL after ASCT remains a strong prognostic factor when adjusted to the other important prognostic factors. These results contribute to the recent concept of autologous graft versus tumor (GVT) effect, which is based on the fact that immune recovery after ASCT in several malignancies (hematological and non-hematological) has been shown to be an independent prognostic factor for survival of cancer patients [85]. The interest in immunotherapy after hematopoietic stem cell transplantation has come from the clinical observation of the GVT effect. The GVT effect has been recognized in the allogeneic context [226-228] where the recognition of allo-major histocompatibility complex (MHC) leads to the killing of residual tumor cells besides graft versus host disease [229-231]. In the autologous setting, patient survival is associated with a rapid recovery of lymphocytes, suggesting that the autologous immune system has the ability to fight residual tumor cells. It is fundamental to understand what the precise role that each subset of lymphocyte plays is, in the immune response to residual tumor cells after autologous hematopoietic transplantation.

A recent report demonstrated that polyclonal immunoglobulin administration is able to increase TCR repertoire diversity in an animal model with contracted T cell repertoire [232]. This could represent a possible approach to be tested in the clinical setting. The possibility of improving repertoire diversity and function of the T cells that populate the immune system after

hematopoietic precursor transplant will allow a better immune function and, probably a lower rate of recurrence of the tumor.

In the case of mantle cell lymphoma is particularly important the knowledge that ALC-15 is a new independent prognosis factor for survival and progression after ASCT. High dose chemotherapy followed by autologous hematopoietic precursor transplant in first remission gives an advantageous to the patients in terms of PFS [212]. However, longer follow up are needed to evaluate the impact of ASCT on overall survival of

MCL patients. ALC-15 seems to be a prognostic factor for disease progression and survival after ASCT in which intervention is worth and possible. Further basic and clinical investigation is needed to understand which specific therapeutic intervention should be used and the magnitude of its benefits.

The retrospective nature of this study limits the response of the fundamental question of which subset of lymphocytes led to the observed increase in survival. Our group has already undertaken a prospective study to address that question. However, we published before that the number of lymphocytes present in the apheresis product infused into the patients correlates positively with the recovery of lymphocytes observed after ASCT in patients with NHL and with survival of these patients [233]. More, retrospectively we found no strong correlation between T or B cells from the apheresis product compared with the ALC at day 15 post-ASCT, but good correlation between NK cells from the apheresis product compared with ALC

at day 15 post-ASCT (CD16+/CD56+/CD3-, r = 0.77) [234]. Although those results were primarily hypothesis generating given the small sample size and the design of the study, it sets the foundation to test immunomodulatory therapeutic approaches aiming early NK recovery.

Given the results of the present study and the previous literature showing that recover of lymphocytes after ASCT is associated with a better survival of patients with cancer [82-84, 86, 87, 218, 219], it is important to further investigate approaches to improve immune reconstitution in the ASCT setting.

6.6. Acknowledgements

The authors thank Tanya Hoskin, Biostatistics, Mayo Clinic Rochester for her help with statistical methods.

CHAPTER VII

Recovery of polyclonal immunoglobulin serum levels to normal levels after autologous stem cell transplantation predicts disease free survival in patients with multiple myeloma

Recovery of polyclonal immunoglobulin serum levels to normal levels after autologous stem cell transplantation predicts disease free survival in patients with multiple myeloma

Cristina Joao MD, MS[1 2], Susan Geyer PhD[3], Svetomir N. Markovic MD, PhD[1], Morie Gertz MD[1], Martha Lacy MD[1], Angela Dispenzieri MD[1], Shagi Kumar MD[1], Suzanne Hayman MD[1], Dennis Gastineau MD[1], Luis F. Porrata MD[1]

[1] Internal Medicine Department, Division of Hematology, Mayo Clinic, Rochester, MN: [2] Hematology Department, Portuguese Institute of Oncology, Lisbon, Portugal; [3] Cancer Center Statistics, Mayo Clinic, Rochester, MN

7.1. Abstract

Background and Aims: The ability of the immune system to recover its components after autologous stem cell transplantation (ASCT) is known to predict survival in patients with neoplasms. Also, our previous work showed that polyclonal immunoglobulin can be used to improve T cell diversity and, consequently, T cell function. The aim of this retrospective study was to understand if the recovery of the polyclonal compartment of immunoglobulin in patients with multiple myeloma (MM) at day 100 (± 40d) after ASCT is a predictor factor for survival in MM patients who were subject to ASCT. **Methods:** From 294 patients with IgG-MM subject to ASCT at the Mayo Clinic, Rochester, MN since October, 1993 until June, 2004, 110 patients were studied. The rational to select this group of patients was the fact that they had levels of serum IgM collected at day 100 ± 40 after ASCT recorded. Progression free survival (PFS) was assessed from the day of the transplant. PFS was studied using Kaplan-Meier curves and the univariate and multivariate analysis were done using proportional Cox regression. The prognostic variables included in the multivariate model were stratified by disease status at the time of immunoglobulin evaluation and comprise recovered IgM at day 100, β2 microglobulin, age, LDH, plasma cell label index and disease status at the time of transplant.

Results: There are 48 females in the study. The median age of the cohort was 58 y (33-75). The median follow up of the study was 34 mo (range, 2 - 140). PFS was significantly higher in the group who recovered normal serum

levels of IgM at D100 compared to the pts who did not. This corresponds to a survival 1.5 longer in the group of patients who recovered normal levels of IgM versus the patients who did not (median: 34 to 23 mo, respectively; p=0.048). By univariate analysis, those who did not recover normal serum levels polyclonal immunoglobulin by day 100 after ASCT had a risk of relapse 1.3 times higher than the risk of the patients who recovered. Multivariate analysis suggests that recover the normal levels of polyclonal immunoglobulin after ASCT is an independent prognostic factor for PFS for MM patients after ASCT.

Conclusions: These results suggest that polyclonal immunoglobulin is a major factor promoting immunity after ASCT.

Keywords: Immunity, Polyclonal Immunoglobulin, Multiple Myeloma, Autologous Stem Cell Transplantation.

Running title: Polyclonal immunoglobulin and immunity in multiple myeloma

7.2. Introduction

MM is the most common primary bone cancer in adults (generally in those older than 50) with an annual U.S. incidence of 4.2 cases per 100,000 people [235].The median survival for multiple myeloma varies from 3 to 4 years [236].

High-dose chemotherapy followed by transplantation has resulted in improvement in response rates and survival compared with conventional therapy, but relapse is nearly universal and not all patients are candidates for this option of aggressive treatment. High-dose therapy with autologous stem cell transplantation (ASCT) has become the treatment of choice for symptomatic eligible patients with multiple myeloma (MM). Several studies have shown that high-dose chemotherapy with autologous stem cell transplantation improves the response rate, event-free survival (EFS), and overall survival (OS) in myeloma over that obtained with conventional chemotherapy [31, 41, 237-239]. In patients who cannot withstand the autologous stem cell transplantation regimen and for treatment of refractory or relapsed disease various chemotherapy regimens are employed as initial therapy in [240].

Immunity in multiple myeloma is known to be impaired [241], what is considered as an additional factor correlated with prognosis and influencing disease progression [242]. Immunological abnormalities, particularly in

patients with advanced disease, have been described in B, T and natural killer (NK) cells. The CD4:CD8 T cell ratio decreases, related mainly to an expansion of the $CD8^+$ lymphocyte subpopulation and a decrease of the $CD4^+$ subpopulation [243, 244]. Other studies point to a variety of T cell dysfunctions [245] [246-248], including an abnormal expression of cell surface molecules, cytokine secretion, high basal activation [249] and an impaired response to mitogens [250]. Thus, it has been suggested that the immune system might play a role in controlling proliferation of the malignant clone and that the disease progresses when the immune system fails [242].
The robustness of the immune system given by the recovery of the number of lymphocytes after autologous hematopoietic stem cell transplant (ASCT) is an independent prognostic factor for survival after ASCT in non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM) [82] and several other hematological and non-hematological neoplasms [83, 86, 87, 218, 251]. Besides the recovery of lymphocytes and its impact on immunity our previous work have also shown that polyclonal immunoglobulin is a self molecule that promotes T cell repertoire diversification and improve T cell function [232, 252] and that it might be used in the clinical to improve immune reconstitution it situations where TCR repertoire diversity is contracted [253].

Here we report that the recovery of polyclonal immunoglobulin compartment to normal serum levels in MM patients after ASCT predicts disease free

survival independently of common considered prognostic factors as β2 microglobulin, age, LDH, plasma cell label index and disease status at the time of transplant.

7.3. Patients and Methods

7.3.1. Patient population. We studied all the patients with IgG MM subject to ASCT at the Mayo Clinic, Rochester, MN since October, 1993 until June, 2004, who had levels of serum IgM collected at day 100 ± 40 after ASCT were included in the study. Response to therapy, relapse, and survival data were updated continuously. All patients gave written, informed consent allowing the use of their medical records for medical research. Approval for the retrospective review of these records was obtained from the Mayo Clinic Institutional Review Board and was in accordance with US federal regulations and the Declaration of Helsinki.

7.3.2. Objective of the Study. The primary objective of this retrospective study was to determine if the recovery of the polyclonal compartment of immunoglobulin in patients with multiple myeloma at day 100 (± 40d) after ASCT is a predictor factor for survival in MM patients who were subject to ASCT.

7.3.3. Prognostic factors. This study included the following prognostic factors: age as a continuous variable, lactate dehydrogenase (LDH) at diagnosis as a continuous variable, recovered of normal IgM at day 100 after ASCT, serum levels of IgM at day 100 after ASCT, β2 microglobulin at diagnosis, CRP at diagnosis, types of conditioning regimen used, bone marrow level of plasma cells, plasma cell labeling index and disease status at the time of transplant.

7.3.4. Conditioning regimens. All patients underwent peripheral blood precursor cells (PBPC) re-infusion after high dose chemotherapy (HDC). The conditioning regimens used as HDC for patients were as follows: 62 patients received Melphalan [200 mg/ m^2], 34 patients received Melphalan [200 mg/ m^2]/Total Body Irradiation, 12 patients received Melphalan/Samarium, 1 patient received Melphalan [140 mg/ m^2] and another patient received Phenylalanine Mustard. Hematologic engraftment was thought to have occurred when the ANC reached 500 cells/μL or more for more than 3 consecutive days.

7.3.5. Response and survival. The stringent criteria specified by the European Group for Blood and Marrow Transplant (EBMT) and published by Bladé et al [254] were used to assess response at day 100 after ASCT. Complete remission (CR) was defined as a stable (more than 6 weeks) 100% of reduction in the serum and urine protein electrophoresis, negative

immunofixation (serum and urine), less than 5% of plasma cells in the bone marrow, normal calcium and stable bone disease. Partial remission (PR) was defined as a steady (more than 6 weeks) ≥50% of reduction in the serum protein electrophoresis, ≥90 % reduction of urine protein electrophoresis, ≥50% reduction in plasma cells in the bone marrow, normal calcium and stable bone disease.

Quantification of serum IgM at day 100 ± 40 after ASCT was done by ELISA (Enzyme Linked Immuno Sorbent Assay).

Progression-free survival (PFS) time was assessed from the initial date of treatment to date of disease progression, or relapse.

7.3.6. Statistical analysis. Progression free survival times were analyzed using the method described by Kaplan and Meier [221]. Differences between survival curves were tested for statistical significance using the 2-tailed log-rank test.

The Cox proportional hazards model [222] was used to evaluate recovery of normal serum levels of polyclonal Ig at day 100 after ASCT in patients with MM subject to ASCT as a prognostic factor for PFS as well as to adjust for other prognostic factors. Multivariate analysis done using Cox regression models tested all variables with a $p < 0.02$ in the univariate analysis. Recovery of normal serum levels of IgM was used as a discrete variable for the study of survival impact and as continuous variable for the analysis of prognostic factors performed by Cox proportional hazards models.

7.4. Results

7.4.1. Patient characteristics

Patient characteristics prior to ASCT are detailed on Table 1.

A total of 110 patients with IgG MM (48 females and 62 males) were included in the study; the median age of the cohort group at transplant was 58 y (range of 33-75). Sixty nine patients (62%) had a MM IgGk, thirty five patients (32%) had MM IgGλ, five patients (4%) had a MM IgGk+λ and one patient (0.9%) had a MM IgGk and IgAλ.

The numbers of prior treatment combination of chemotherapy used varied from 1 to 3; the majority of the patients (79%) were subject to just one type of chemotherapy regimen before ASCT. Prior treatments included Dexamethasone; Thalidomide; VAD (Vincristine, Adriamicin, Dexamethasona); VBMCP (Vincristine, Melphalan, BCNU, Cyclophosphamide, Prednisone); CHOP (Cyclophosphamide, Adriamicin, Vincristine, Prednisone), alone or in combination.

PFS was assessed from the day of ASCT. The median follow-up was 34 months (2-140 months).

Table 1 – Patients characteristics (N=110)

Characteristics	
Age	**Median, Range** 58,5, 33-75
Sex	**Number of patients (%)**
Females	48 (44%)
Males	62 (56%)
Cytogenetics	
Normal	91(82%)
13 inversion	1 (0.9%)
Complex	8 (7%)
Other	10 (9%)
Number of prior treatments	
1	87 (79%)
2	19 (17%)
3	4 (4%)
Conditioning Regimen	
Melphalan/ Total Body Irradiation	34 (31%)
Melphalan 200 mg/m^2	62 (56%)
Melphalan 140 mg/m^2	1 (0.9%)
Melphalan – SAM	12 (11%)
L-Phenylalanine Mustard	1 (0.9%)
Disease Status at ASCT	
Relapse	40 (36%)
Plateau/Response	70 (64%)

7.4.2. Progression and survival

Univariate analysis of prognostic factors

At day 100 after ASCT, 96% (106/110) of the patients were in response (19 in complete responses and 87 in partial responses) and 4% (4/110) of the patients were in progression. All the patients who were in progression at day 100 after ASCT did not recovered normal levels of IgM at that date.

The median PFS was significantly better for patients who recovered normal levels of IgM (n = 30) vs those who did not recovered (n = 80) normal IgM levels at day 100 after ASCT (23 months vs 35 months, $p < 0.048$, respectively) (Figure 1). Performing a similar analysis only in the sub group of patients who were at response (complete or partial) at day 100 after ASCT, the patients who recovered normal levels of IgM continue to have a longer PFS compared to those who did not recovered normal levels of IgM (24 months vs 34 months, $p < 0.07$, respectively) (Figure 2).

From all the prognostic factors studied here, several were identified as significant for PFS (Table 2). Having recovered normal values of serum levels of IgM in patients with IgG MM at day 100 after ASCT, was identified as a significant prognostic factor for PFS (HR = 0.76, $p < 0.04$), meaning that the patients who recovered the polyclonal compartment of Ig at day 100 after ASCT have a risk of relapse that is approximately a third lower for the patients who do not recovered normal levels of serum IgM. Performing a similar analysis analyzing the impact of the continuous numeric value of IgM

at Day 100 (continuous variable) for PFS, the HR obtained is 0.99, p=0.02; meaning that for each increased unit of IgM (mg/dl) at day 100 after ASCT the risk of relapse decrease 1%.

Other factors were found to impact on progression free survival in our cohort of MM patients (Table 2), namely β2 microglobulin at diagnosis, LDH at diagnosis, percentage of bone marrow plasma cells at diagnosis, plasma cell labeling index, disease status at diagnosis and having aneuplodia in the cytogenetics evaluation of diagnosis.

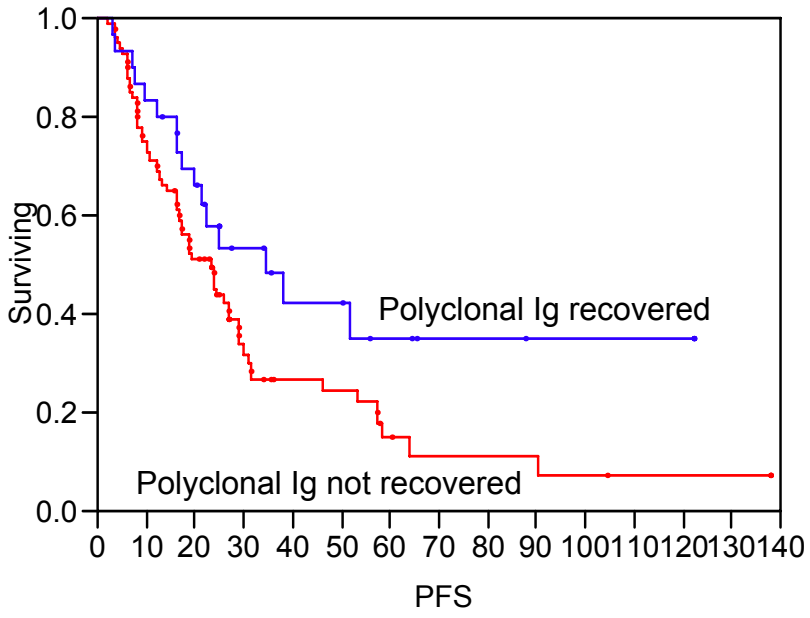


Figure 1 - Progression free survival analysis of IgG MM patients after ASCT. Analysis was performed based on having or not recovered normal serum levels of IgM at day 100 after ASCT. The patients who recovered normal levels of polyclonal Ig after ASCT achieved a longer PFS, p=0.048.

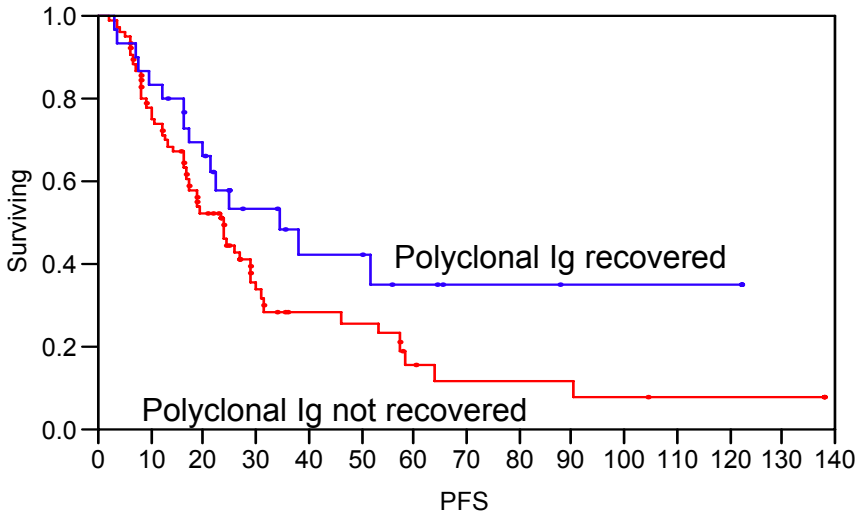


Figure 2 - Progression free survival analysis in the sub group of IgG MM patients who were in response at day 100 after ASCT. The patients who recovered normal levels of polyclonal Ig after ASCT achieved a longer PFS compared to those who did not recover normal levels of IgM, p=0.07.

7.4.3. Multivariate analysis of prognostic factors

Cox regression models for multivariate analysis of the prognostic factors highly significant in the univariate analysis were developed. Having recovered normal serum levels of IgM at day 100 after ASCT is an independent prognostic factor for progression free survival in a model that considered B2 microglobulin, recover IgM serum levels, percentage of bone marrow plasma cells, plasma cell labeling index and having an aneuploidy cytogenetics versus normal (Table 3).

Table 2 – Univariate analysis for PFS on patients with IgG MM subject to ASCT.

Prognostic factors	*Progression free survival*		
	*HR**	*95% CI*	*p value*
Age	1.00	0.99-1.03	0.99
β2 microglobulin	**1.23**	**1.07-1.39**	**0.004**
LDH at diagnosis	**1.0**	**1.0-1.0**	**0.05**
Recovered normal levels IgM at D100 vs not recovered IgM	**0.76**	**0.57-0.99**	**0.04**
Level of serum IgM at Day 100	**0.99**	**0.97-0.99**	**0.02**
Bone marrow plasma cells	**1.02**	**1.00-1.03**	**0.004**
CRP at diagnosis	1.19	0.92-1.47	0.16
Conditioning regimen	0.87	0.55-1.38	0.55
Disease status at the transplant (Plateau/Response vs Relapse)	**1.57**	**0.99-2.47**	**0.05**
Plasma Cell Labeling Index	**1.34**	**1.15-1.53**	**0.0009**
Aneuploid vs normal	**0.58**	**0.49-0.78**	**0.0004**

Table 3 – Multivariate analysis for PFS on patients with IgG MM subject to ASCT.

Prognostic factors	***Progression free survival***		
	HR*	***95% CI***	***p value***
β2 microglobulin	**1.21**	**1.01-1.44**	**0.04**
Bone marrow plasma cells	0.99	0.97-1.01	0.63
Level of serum IgM at Day 100	**1.30**	**0.99-1.76**	**0.05**
Plasma Cell labeling Index	1.21	0.99-1.45	0.06
Aneuploid vs normal	**2.56**	**1.31-4.78**	**0.007**

7.5. Discussion

Multiple myeloma is a disease where immune deregulation is manifest. Several are the clinical situations that illustrate this phenomenon during the course of the disease. Myeloma patients have higher risk of infection [255, 256], abnormal B cells function and hypogamaglobulinemia [247], altered lymphocytes sub population numbers [245], decrease TCR repertoire diversity [257], among other changes of the immune system that contribute to the elevated number of infections on this patients' population [258, 259]. Some of these authors suggest that myeloma patients, who are at increased risk of serious infection, can be identified and may benefit from replacement immunoglobulin therapy to reduce the risk of infection. Chapel H et al., from the UK Group for Immunoglobulin Replacement Therapy in Multiple Myeloma, reported significant benefit on the use of intravenous immunoglobulin as prophylaxis against infection in plateau-phase multiple myeloma in a multi center randomized trial [260].

Given all these immune alterations related with this particular disease, it is key to guarantee a high-quality immune recovery after myeloablative treatments as ASCT, as the ability to fight minimal residual disease, what is highly prevalent in MM patients after autologous transplantation [261]. Also, providing that intravenous immunoglobulin protects against life threatening

infections and significantly reduces the risk of recurrent infections, it is reasoning to expect that recover of polyclonal compartment of Ig during immune reconstitution after autologous transplantation in MM patients would impact on relapse risk and/or patient survival.

At baseline and after treatment approaches as ASCT, the levels of immune cells and the rate of immune reconstitution were identified as determinant factors affecting survival of these patients [82, 85, 262].

In Porrata et al's studies the immune reconstitution after ASCT was analyzed using the absolute number of lymphocytes as a surrogate of the immune system robustness. Hofer at al. reported as an abstract, that the recovery of polyclonal immunoglobulin synthesis after ASCT in MM patients was a prognostic factor for event-free survival [263]. Here we studied the recovery of IgM levels in IgG MM patients subjected to ASCT. Our results suggest that the recovered of the polyclonal compartment of immunoglobulin during immune reconstitution after ASCT in patients with MM is crucial to vigor the immune system detecting minimal residual disease, decreasing the risk of relapse. This effect seems independent of other predictor factors such as b2 microglobulin and aneuploidia at cytogenetics analysis. The progression free survival of patients who recovered normal levels of IgM at D100 after ASCT is prolonged in approximately one year compared to those who did not recovered normal levels of IgM at D100, even in the sub analysis of patients who were in response at D100 after ASCT. The power of this analysis is somehow limited by the fact that only the serum levels of recovered IgM at

D100 after transplant were analyzed here. However, it is predictable that a time dependent analysis of recovered serum levels of IgM, collected since the date of transplant until the end of follow up, would significantly increase the statistical differences already detected by this analysis at one time point, i.e., day 100 after ASCT.

The fact that treatment with intravenous immunoglobulin is used widely in clinical practice to treat infections [93] and control inflammation [91], and given the crescent evidence that is useful during immune reconstitution [252, 253], it seems reasonable to expand measures to increase serum levels of polyclonal immunoglobulin in myeloma patients who underwent autologous stem cell transplantation.

CHAPTER VIII

Host immune competence predicts a superior progression-free survival in non-Hodgkin's lymphoma patients treated with Rituximab and Interleukin-12

Host immune competence predicts a superior progression-free survival in non-Hodgkin's lymphoma patients treated with Rituximab and Interleukin-12

Cristina João MD, MS[1 2], Luis F. Porrata MD[1], Thomas Witzig MD[1], Paul Kurtin MD[3],Ivana Micallef MD[1], Svetomir N. Markovic MD, PhD[1], Charles Erlichman MD[4], Anne Novak PhD[1], Stephen Ansell MD, PhD[1]

[1]Division of Hematology/Department of Internal Medicine, Mayo Clinic College of Medicine, Rochester, MN, 55905.
[2]Department of Hematology, Portuguese Institute of Oncology, Lisbon, Portugal
[3]Department of Pathology, Mayo Clinic College of Medicine, Rochester, MN, 55905
[4]Medical Oncology, Mayo Clinic College of Medicine, Rochester, MN, 55905

This study was funded by the grants from the National Institutes of Health (CA92104 and CA25224) and from Fundação para a Ciência e Tecnologia, Lisbon, Portugal (BD 6797/2001).

Manuscript in preparation, 2007

8.1. Abstract

Background/Objective: Rituximab immune-mediated mechanisms of action suggest that it efficacy depends on host immunocompetency. Therapies, such as IL-12, that stimulate the immune system may therefore increase rituximab efficacy. We hypothesized that a stronger host immune system at the time of rituximab would result in better clinical outcome. Herein, we present data supporting that host immune-competence, analyzed by absolute lymphocyte count (ALC) and lymphocyte subsets in the peripheral blood, predicts a superior survival in non-Hodgkin lymphoma (NHL) patients treated with rituximab-based regimens.

Study Methods: We studied 41 NHL-patients participating in a Phase I study of interleukin-12 (IL-12) and rituximab.

Results: The median follow-up was 42 months. Patients with ALC ≥ 0.9 x 109/L experienced superior progression free survival (PFS) compared to patients with ALC < 0.9 x 109/L prior to rituximab therapy (21 vs 4 months, $p < 0.001$). Patients with CD4 count ≥ 500 cells/μl also experienced a superior PFS compared to patients with CD4 count < 500 cells/μl (21 vs 9 months, $p < 0.0062$). Multivariate analysis demonstrated ALC to be an independent prognostic factor for PFS.

Interpretation and conclusions: Our data denote the value of an immune competent host in predicting the efficacy of rituximab therapy in NHL.

Keywords: Immunity, Lymphocyte number, Rituximab therapy, Non Hodgkin lymphoma.

Running title: Response to Rituximab depends on the immune system status

8.2. Introduction

The exact in vivo mechanism of action of rituximab is not fully elucidated. However, the cytotoxic effects of rituximab on CD20-positive malignant B cells appear to involve complement-dependent lysis [264], complement-dependent cellular cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) [265], induction of apoptosis [266] and sensitization of tumor cells to the effects of chemotherapy through the modulation of molecular signal pathways [267]. Thus, rituximab induces responses by inducing apoptosis but also by recruiting immune cells to help in the lysis of malignant cells.

As the vast majority of the proposed mechanisms of action of rituximab are immune-mediated, we predicted that the efficacy of rituximab therapy would depend on the immunocompetency of the host. Thus, assessing and predicting patient immunity before treatment with rituximab would allow a better selection of the patients who would benefit from this treatment in terms of clinical outcomes.

Interleukin-12 (IL-12) is a cytokine that has been shown to have a profound effect on T and natural killer (NK) cell function [268, 269] and that increases ADCC [55]. IL-12 used alone as a therapeutic agent in cases of relapsed and refractory hematological malignancies has shown promising results [51, 270, 271]. This and the fact that the mechanism of action of IL-12 suggested that it could have an additive anti-tumor effect when given with rituximab, formed

the basis for combining rituximab and IL-12 in an investigational protocol to treat non-Hodgkin lymphoma (NHL) [51, 272].

The absolute lymphocyte count (ALC) recovery ≥ 500 cells/μL at day 15 (ALC-15) after autologous stem cell transplantation (ASCT) has been reported as a powerful and independent prognostic factor for clinical outcomes for patients with NHL [82, 216, 217], Hodgkin lymphoma [84, 216], multiple myeloma [82], acute myelogenous leukemia [218], amyloidosis [83], and breast cancer [86/, 219]. Furthermore, the time-dependent kinetics to achieve an ALC ≥ 500 cels/μL after ASCT also predicts overall survival (OS) and progression free survival (PFS) after ASCT in NHL [273], corroborating the importance of lymphocyte recovery as a surrogate of immune recovery.

To assess the significance of an immune competent host in predicting the efficacy of rituximab therapy we conducted a retrospective study of NHL patients treated with Interleukin-12 and rituximab and assessed lymphocyte numbers, lymphocytes subsets, response rate and PFS.

8.3. Patients and Methods

8.3.1. Patient population. We studied forty-one NHL patients that participated in our Phase I interleukin-12 plus Rituximab study [51]. The rationale for selecting this group of patients was that besides having a baseline ALC count performed, all patients required baseline flow cytometry analysis providing lymphocyte subset data prior to treatment. Response to therapy, relapse, and survival data were updated continuously. All patients gave written, informed consent allowing the use of their medical records for medical research. Approval for the retrospective review of these records was obtained from the Mayo Clinic Institutional Review Board and was in accordance with US federal regulations and the Declaration of Helsinki.

8.3.2. End points. The primary end-point of the study was to assess whether the ALC count prior to rituximab/IL-12 therapy predicted PFS and the second end-point was to identify which lymphocyte subset in the ALC was responsible for affecting PFS after rituximab/IL-12 therapy.

8.3.3. Lymphocyte subset analysis. Immunophenotyping of mononuclear cells was performed using a two-color direct staining of cells with mouse monoclonal antibodies. Fluorescein isothiocyanate- or phycoerythrin-coupled monoclonal antibodies (Becton Dickinson PharMingen, San Diego, CA, USA) were directed at human CD3, CD4, CD8, CD16/CD56, CD19. Phenotypic analysis was performed with a FACStar PLUS flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with CONSORT 40 software (Becton Dickinson Biosciences, San Jose, CA, USA). Fluorescein isothiocyanate- and phycoerythrin-coupled isotype-matched immunoglobulins were used as isotypic controls.

8.3.4. Prognostic factors. This study included the following prognostic factors: international age-adjusted prognostic index (IPI) [220] (age (≥60 vs <60), lactate dehydrogenase (LDH) > normal, performance status (PS-ECOG) (≥2 vs <2), extranodal sites (≥2 vs <2) and stage (III/IV vs I/II)), in addition to the number of lymphocytes (ALC), CD3, CD4, CD8, CD19, CD16/56, number of previous treatments (≥2 vs <2), the dose used of IL-12, and the histological subtype of lymphoma. The ALC and numbers of lymphocytes subset were determined immediately prior to the treatment. In the case of follicular lymphomas Follicular lymphoma international prognostic index (FLIPI)[274] was also included in the analysis.

8.3.5. Response and survival. Response criteria were based on the guidelines from the NHL International Workshop [220]. Complete remission (CR) was defined as complete regression of all measurable disease including radiologically demonstrable disease, BM involvement, or peripheral blood involvement. Partial remission (PR) was defined as a reduction in the sum of the products of measurable lesions' longest diameters and perpendicular diameters of 50% or greater, with a 30% or greater decrease in hepatomegaly or splenomegaly (measured from the costal margin), if there was previous known liver or spleen involvement. Disease progression was defined as a 25% or more increase in the sum of the products of the longest diameter and its perpendicular diameter of measurable lesion(s) from the pre-study measurement, the appearance of new lesions, or a 2-cm increase in spleen or liver size due to lymphoma.

Progression-free survival (PFS) time was assessed from the initial date of treatment to date of disease progression, or relapse.

8.3.7. Statistical analysis. PFS times were analyzed using the method described by Kaplan and Meier [221]. Differences between survival curves were tested for statistical significance using the 2-tailed log-rank test. ALC and CD4 were assessed as continuous variable and dichotomized, based on the optimal

cut-off point based on the log-rank statistic. The ALC and the CD4 values analyzed to identify the optimal cut-off point included a range from 0.37-5.62 x 10^9/L and 80-2072 cells/µl, as these values were between the 25% and 75% quartiles for ALC and CD4, respectively. The Cox proportional hazards model [222] was used to assess ALC and lymphocyte subsets as a prognostic factor for PFS as well as to adjust for other prognostic factors. Multivariate analysis done using Cox regression models tested all variables with a $p < 0.008$ in the univariate analysis. The p-value cutoff to move forward a factor from the univariate into the multivariate was selected based on the use the Bonferroni's correction procedure for multiple comparisons with a small number of events.

In addition to the evaluation of ALC and its prognostic significance for PFS, its utility as a marker for CD4 was also assessed. The choice of the optimal cut-off of ALC was based on its utility as a marker for CD4 using ROC curves and area under the curve (AUC) analysis. Prediction of CD4 numbers was explored further in logistic regression models, univariately assessing continuous and dichotomized values of ALC as well as the other potential prognostic factors described above. Pearson's chi-square analysis was used to determine relations between nominal variables; the Wilcoxon rank-sum tests or the Kruskal-Wallis rank-sum tests were used to determine associations between continuous variables and categories, and Spearman correlation coefficients were used to evaluate correlation for continuous

variables. All p values represented were 2-sided, and statistical significance was declared at $p < .05$.

8.4. Results

8.4.1. Patient characteristics

Patient characteristics prior to treatment with Rituximab/IL-12 are detailed on table 1. Of the 41 NHL patients studied, 20 were females. The median age of the cohort was 53 years (range: 34-84). All the patients had performance status (ECOG) 0 or 1. At diagnosis, twenty-four patients had stage 4 disease, 11 patients in stage 3, 5 patients stage 2 and 1 patient had stage 1 disease. The numbers of prior treatments varied from 0 to 6. PFS was assessed from the initial date of treatment. The median follow-up was 42 months (1-62 months). The median ALC prior to rituximab/IL-12 therapy was 0.97 x 10^9/L (range, 0.37-5.62 x 10^9/L).

Table 1 – Patients characteristics (N=41)

Characteristics	
Age	**Median, Range**
	53, 34-84
Sex	**Number of patients (%)**
Females	20 (49%)
Males	21 (51%)
Lymphoma histology	**Number of patients (%)**
Follicular	21 (51%)
Diffuse large cell	10 (24%)
Mantle cell	5 (12%)
Lymphoplasmacytic	2 (5%)
Lymphocytic	2 (5%)
T-cell rich B-cell	1 (3%)
Number of prior treatments	**Median, Range**
	1, 0-6
Treatment	**Number of patients (%)**
Rituximab* alone	6 (15%)
Rituximab* plus IL-12**	
IL-12 30 ng/Kg	6 (15%)
IL-12 100 ng/Kg	9 (22%)
IL-12 300 ng/Kg	13 (31%)
IL-12 500 ng/Kg	7 (17%)
Lymphocyte subset prior to treatment/μL	**Median, Range**
CD4+ T cells	359, 80-2072
CD8+ T cells	304, 65-2677
NK cells	204, 32-1338
B cells	87, 3-1027

* Rituximab 375 mg/m^2 i.v. weekly for 4 weeks
**IL-12 was done s.c. twice weekly at different doses as detailed, for a maximum of 24 weeks.

8.4.2. Progression and survival

Univariate analysis of prognostic factors

At the time of this analysis, 78% (32/41) patients had progressed and 41% (17/41) patients had died. ALC, as a continuous variable, was identified as a prognostic factor for PFS (HR = 0.99, $p < 0.001$) (Table II). The use of ALC $\geq 0.9 \times 10^9$/L as a cutoff value was selected as it yielded the highest difference in the survival by χ^2 when different cut points were analyzed from 0.37-5.62 x 10^9/L. The median PFS was significantly better for patients with an ALC $\geq 0.9 \times 10^9$/L (n = 23) vs an ALC $< 0.9 \times 10^9$/L (n = 18) from the time of rituximab/IL-12 therapy (21 months vs 4 months, $p < 0.001$, respectively) (Figure 1). At the study date, 14/23 (60%) patients with ALC $\geq 0.9 \times 10^9$/L had progressed while all the 18 (100%) patients with ALC $< 0.9 \times 10^9$/L had progressed, $p< 0.003$.

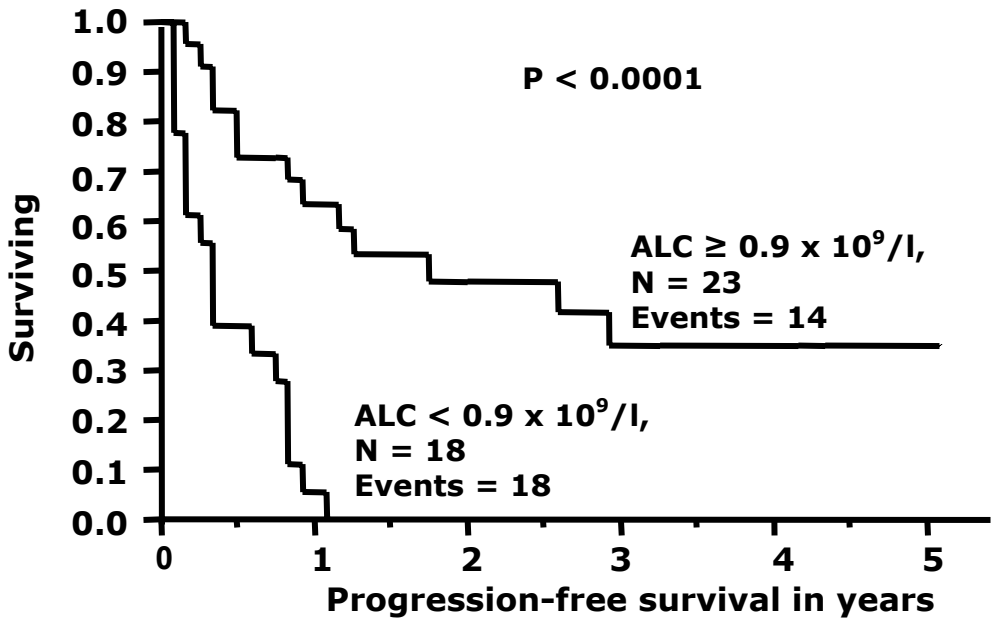
The lymphocyte subset analysis revealed that CD4, as continuous variable, was also identified as a prognostic factor for PFS (HR = 0.99, $p = 0.02$) (Table II). The use of CD4 $\geq$ 500 cells/μl as a cut-off value yielded the highest difference in survival by χ^2 when different cut points were analyzed from 80-2072 cells/μl. The median PFS was significantly better for patients with a CD4 $\geq$ 500 cells/μl (n = 19) vs a CD4 < 500 cells/μl (n = 22) from the time of rituximab/IL-12 therapy (21 months vs 9 months, $p < 0.006$, respectively) (Figure 2). At the time of this analysis, 9 of the 16 patients with CD4 $\geq$ 500 cells/μl (56%) had progressed while 22 of 25 patients with CD4 < 500 cells/μl (88%) had progressed, $p< 0.0005$.

In addition, PFS was not affected by the different doses of IL-12 administered ($p = 0.18$). However, in patients that received IL-12 doses of 100ng/kg, 300ng/kg, and 500ng/kg, a superior PFS was observed in those patients with an ALC $\geq 0.9 \times 10^9$/L compared to patients with an ALC $< 0.9 \times 10^9$/L [100ng/kg group (median: 21 vs 10 months, $p < 0.04$); 300ng/kg group (median: 31 vs 4 months, $p < 0.01$); and 500ng/kg (median: not reached vs 7 months, $p < 0.02$)].

Table 2 – Univariate analysis for PFS in patients with NHL subject to treatment with rituximab/IL-12.

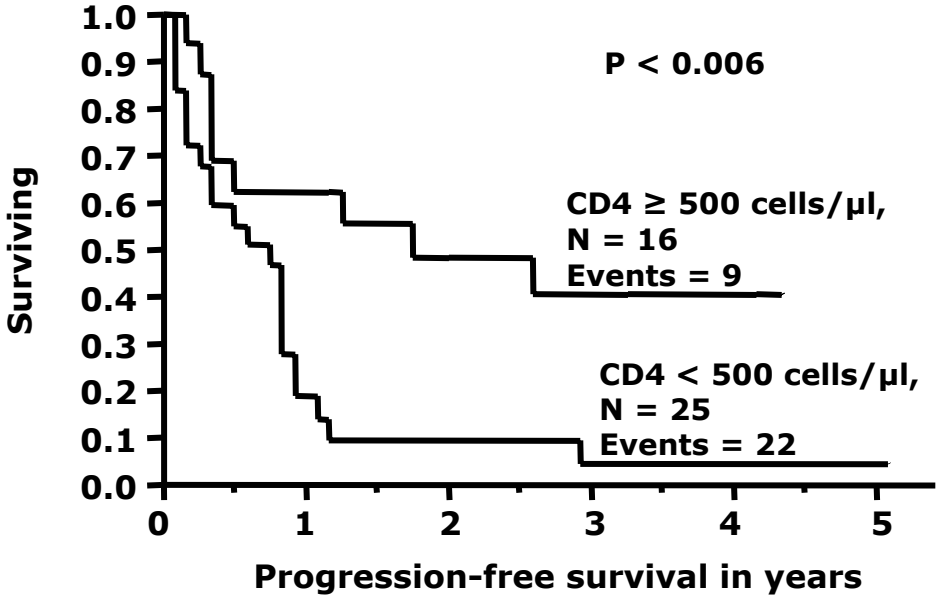
Prognostic factors	*Progression free survival*		
	HR	*95% CI*	*p value*
Age	1.00	0.98-1.03	0.74
LDH (normal vs > normal)	**3.62**	**1.45-8.34**	**0.008**
Number of prior treatments (≥ 2 vs <2)	**5.8**	**2.5-15.3**	**<0.0001**
Stage (III/IV vs I/II)	0.94	0.36-3.21	0.90
Extra-nodal sites (≥ 2 vs <2)	2.48	0.83-6.09	0.10
Histology (follicular vs other)	1.32	0.93-1.91	0.12
Disease status prior to rituximab (NR vs CR/PR)	**2.25**	**1.44-3.45**	**0.0006**
IL-12 dose*	**0.67**	**0.46-1.00**	**0.05**
ALC/μL prior to rituximab	**0.99**	**0.998-0.999**	**0.001**
NK cells prior to rituximab	1.00	0.99-1.00	0.96
CD4+ T cells prior to rituximab	**0.99**	**0.997-0.999**	**0.02**
CD8+ T cells prior to rituximab	0.99	0.99-1.00	0.48
CD19+ cells prior to rituximab	0.99	0.99-1.00	0.20

HR – Hazard ratio.
95% CI – 95% Confident interval.
***** Natural Log transformation.



Number at risk						
ALC ≥ 0.9 x 10^9/l	23	14	9	8	4	1
ALC < 0.9 x 10^9/l	18	1	0	0	0	0

Figure 1 – Progression free survival for 41 patients with non Hodgkin lymphoma from the time of rituximab/IL-12 therapy as a function of recovery of absolute number of lymphocytes.



Number at risk						
CD4 ≥ 0.5 x 10^9/l	16	10	7	4	3	
CD4 < 0.5 x 10^9/l	25	6	6	2	2	1

Figure 2 – Progression free survival for 41 patients with non Hodgkin lymphoma from the time of rituximab/IL-12 therapy as a function of recovery of CD4 lymphocyte.

Multivariate analysis of prognostic factors

Only prognostic factors that fulfilled the criteria of a $p < 0.008$ were analyzed in the multivariate analysis. This value was found using the Bonferroni's correction for multiple comparisons. ALC remained an independent prognostic factor for PFS (HR = 0.56, $p < 0.03$) when compared to LDH, number of prior treatments and disease status prior to rituximab (Table 3).

Table 3 – Multivariate analysis for PFS in patients with NHL treated with rituximab/IL-12.

Prognostic factors	***Progression free survival***		
	HR	***95% CI***	***p value***
LDH (normal vs > normal)	1.44	0.87-2.37	0.15
Number of prior treatments (≥ 2 vs <2)	2.47	0.86-7.45	0.09
Disease status prior to rituximab (NR vs CR/PR)	**1.85**	**1.14-2.98**	**0.01**
ALC prior to rituximab	**0.56**	**0.27-0.96**	**0.03**

8.4.3. Sub-analysis of follicular lymphomas

Due to the heterogeneity of the histologies analyzed in this study, we analyzed the role of ALC and CD4 in patients with follicular lymphomas. In the 21 patients with follicular lymphoma, the univariate analysis demonstrated that CD4 numbers (HR = 0.996, $p < 0.008$) and ALC (HR = 0.35, $p < 0.002$) were prognostic factors for PFS**.** Besides these two parameters, LDH at diagnosis (HR=5.72, $p = 0.02$), the number of prior treatments (HR=5.01, $p = 0.01$), disease status prior to rituximab (HR=2.23, $p = 0.03$), FLIP>2 (HR=3.57, $p = 0.045$) and IL-12 dose (HR=0.32, $p = 0.003$), were also predictive factors for PFS. In contrast, for non-follicular lymphomas, LDH and IL-12 dose were not significant predictors for PFS ($p = 0.12$ and $p = 0.75$, respectively). In addition, stage at diagnosis and presence of extra nodal disease were statistically significant as predictors for PFS for non-follicular lymphomas ($p = 0.04$ and $p = 0.01$, respectively).

The multivariate analysis shows, in the sub-group of patients with follicular lymphoma, that CD4+ T cells counts prior to rituximab (HR = 0.996, $p < 0.03$) is a significant independent predictor factor for PFS after rituximab, adjusting for the Follicular Lymphoma International Prognostic Index at the time of treatment.

8.4.4. The role of ALC on CD4 counts

Due to the fact that CD4 counts are not routinely ordered in every patient treated with rituximab, we assessed the utility of ALC (obtained from the available and standard complete blood cell count) as a marker of CD4. ROC curves and AUC analysis showed that ALC was a significant marker for CD4 (AUC =0.82, $p < 0.0009$) and a sensitivity of 95% with a specificity of 68%. With regard to the association between ALC and CD4, these dichotomized variables were found to be significantly correlated with each other ($p < 0.0005$) as were their continuous counterparts (r_s=0.65, $p < 0.0001$). Logistic regression models for predicting CD4 further indicate that ALC is significantly correlated with this clinical outcome ($p < 0.0025$). These results increase the generability and external validity of this study.

Another significant factor for CD4 in the univariate setting was the number of prior treatments ($p < 0.0007$), as patients that received more than two prior treatments had less CD4 count compared to those who did not prior to rituximab therapy (median: 0.77 vs 1.38 cells/μl, $p < 0.04$, respectively). When these factors were accounted for in a multivariate logistic regression model, ALC remained significantly correlated with the CD4 count ($p < 0.01$). An ALC $\geq 0.9 \times 10^9$/L was associated with an adjusted odds ratio for CD4 count of 6.74 (95% CI, 1.49-38.1). Despite the different histologies, no association was identified between histology and ALC (as continuous variable, $p = 0.77$, or dichotomized variable, $p = 0.76$) or CD4 count (as continuous variable, $p = 0.77$, or dichotomized variable, $p = 0.76$). In addition, no

association was identified between histology and lymphocyte subsets, as continuous variables (CD3, p = 0.1; CD8, p = 0.23; CD19, p = 0.55, and NK cells, p =0.11).

8.5 Discussion

The underlying immunologic mechanisms of rituximab therapy suggest that the host immune status plays an important role of in the therapeutic efficacy of rituximab [36]. Therefore, we set out to investigate whether the host immune system, as defined by the ALC (as a surrogate marker for host immune status) and lymphocyte subsets, affects the clinical outcome of patients treated with rituximab. We analyzed patients that participated in a phase I trial of rituximab and IL-12 as it was mandatory in this study to obtain baseline lymphocyte subset numbers besides ALC prior to therapy.

This study shows that patients with a higher ALC at the time of rituximab therapy experienced better progression-free survival compared to those who did not. This finding concurred with a recent report showing that ALC was an independent prognostic factor for event-free survival in patients with follicular or mantle cell lymphoma treated with single agent rituximab [275]. Our study differed in the fact that we identified that not only ALC is a prognostic factor for progression-free survival, but also that patients with higher numbers of CD4 cells in the peripheral blood have a superior clinical outcome. This finding is significant because the presence of activated CD4+ T-cells infiltrating the tumor bed of lymphoma patients has been reported to affect survival [276]. The authors argued that a possible mechanism leading to the better outcome of patients with higher numbers of CD4+T-cells

infiltrating the tumor bed is that CD4+ T-cells may be responsible for stimulating antigen-presenting cells, thereby up regulating the immune response; and enhancing the anti-tumor immune response. Further research of sub-sets of CD4+ T cells, namely characterizing TH1 or TH2 cytokine profile production, and also regulatory phenotype and activity of CD4 T cells may better elucidate the precise immunological role of CD4+ T cells prior to treatment with rituximab-combined antineoplastic treatments in the clinical outcome of NHL patients.

Patients in this study were also received IL-12 with the intention of enhancing the immune response against the tumor. Regarding the dose of IL-12 concomitantly administered with rituximab therapy, which was found to be associated with PFS, it is important to comment on the way this analysis was performed. The analysis of the dose of IL-12 used was done by comparing the natural logarithm of the dose increment of IL-12 as predictor factor for PFS, by Cox analysis. This type of analysis allowed us to compare the importance of using higher increments versus smaller increments of IL-12 dose to achieve a better clinical response. Because, there was no escalation of dose within the same patient in the study, this result suggests that patients who started IL-12 at a higher dose achieved a better clinical outcome compared to patients who were treated with IL-12 at smaller doses. However, the Phase I trial was not powered for this specific analysis [51]. This predictor factor was found to be dependent on the ALC prior to diagnosis, suggesting that IL-12 treatment itself achieve better results in

patients whose immune system is in a better shape, as defined by the number of lymphocytes in the peripheral blood. This report gives grounds to hypothesize that the clinical results of NHL immunotherapy depend on the robustness of the patient's immune system and predicts that very immune depressed patients may not be the best candidates for this type of approach. This is exemplified by the fact that the number of prior treatments was a poor prognostic factor for progression-free survival in this study. Patients who received more than 2 prior treatments had lower peripheral CD4 count and ALC prior to rituximab therapy. Thus, the immunosuppressive effect of chemotherapy should be taking into consideration in the future planning of immunotherapeutic studies in the light of these findings.

The routine lymphocyte subset analysis by flow cytometric analysis in patients treated with rituximab is not standard of practice and the application of this technique might be hampered by its cost and restricted availability in clinical practice. Thus, we studied ALC as a surrogate marker for CD4 counts. This study showed a strong correlation between ALC and CD4, suggesting that ALC is a good marker of the immune status of the patient. Since ALC is obtained from the standardized complete blood count, ALC is a more practical, less costly, and more available prognostic factor to assess the immune status of the patient prior to rituximab therapy.

This study included NHL patients who participated in our Phase I interleukin-12 plus rituximab study and that had flow cytometry analysis performed immediately before the treatment. This selection of patients limited the

potential findings of this study as only a small number of patients were selected. Besides this limitation, this study highlights the fact that a competent immune system is required for clinical benefit with rituximab therapy in NHL patients. More studies are warranted to analyze the status of the host immune system as a potential variable affecting outcome as immunological therapies are tested in the clinic setting.

Chapter IX

Discussion, General Conclusions and Future Directions

Chapter IX

Discussion, General Conclusions and Future Directions

The data presented in this dissertation originated several novel scientific conclusions and, may point to a new paradigm concerning the way immunologists and physicians perceive B and T lymphocyte interdependence and the immune system web of relationships.

In collaboration with Dr. Jeffrey Platt's group at the Mayo Clinic, I developed a new quantitative method to directly assess lymphocyte diversity and applied this method to B and T cell populations. This novel technique uses gene chips arrays and its rational, demonstrated in Chapter III, is based on the fact that the frequency of hybridization of nucleic acids coding for lymphocyte receptors to the oligonucleotides on a gene chip varies in direct proportion to diversity. We applied the technique to detect changes in lymphocyte diversity in mice with known B cell alterations and in people with known T cell repertoire defects. This approach is the first to provide a direct analysis of lymphocyte receptor diversity and has already facilitated fundamental studies of the adaptive immune system and clinical efforts to assess immunological diseases.

This work shows that B cells and polyclonal immunoglobulin are essential elements driving T cell receptor diversity generation and, subsequently, peripheral T cell function. As demonstrated in Chapter IV, polyclonal immunoglobulins have an essential role in the development of T cells in

the thymus as promoters of TCR diversification. This may happen through the presentation of diverse immunoglobulin's peptides by B cells and other antigen presenting cells existing in the thymus, to the developing T cell during the process of TCR diversification. The result is to promote positive selection of thymocytes and to decrease death by neglect. This hypothsis is reinforced by the finding of an increased apoptosis in the thymic cortex of mice that lack B cells or immunoglobulin. Since apoptosis in the thymic cortex of wild type mice is comparable to apoptosis detected in mice that produce serum immunoglobulin but have few B cells (μMT mice), our results indicate that serum immunoglobulin also promotes thymocyte survival besides its effect on promoting TCR diversification.

These observations may have great implications to the way scientists and physicians perceive the immune system and its deregulations. Thus help to elucidate the phenotype of B cell-deficient human individuals and suggest a potential approach to immune reconstitution using immunoglobulin.

As a consequence of these results, we decided to examine the role of immunoglobulin in the function of peripheral T cells. Polyclonal immunoglobulin is used for the treatment of several diseases. However, the impact of its use on the function of the T cell compartment needs further characterization. With this purpose we developed mouse models that allow the investigation of the role of B cells and immunoglobulin to the function of

T cells at the periphery.

In vivo testing of T lymphocyte function was performed using H-Y incompatible skin grafts; the survival time of the grafts was used as a measurement of the function of T cell lymphocytes. Skin grafts are rejected by CD4+ and CD8+ T cells, so an impairment of T lymphocyte function would allow a longer survival of the grafts. Rejection of H-Y incompatible skin graft in mice with oligoclonal B-cells and in mice lacking B-cells was significantly delayed compared to wild type mice, indicating that the presence of B cells and/or immunoglobulin are essential to an enhanced T cell function. The assessment of TCR V β diversity on these mice (all strains with the same MHC background) demonstrated that the presence of B cells or polyclonal immunoglobulin was determinant for the diversity of the T cell repertoire.

To verify these rational and results, we induced a reduction of the TCR V β diversity by thymectomy in wild type mice and observed a significant increase of the survival of H-Y incompatible skin grafts. Also, the reconstitution of T-cell diversity in mice with oligoclonal B-cells with immunoglobulin Fab fragments significantly decreased survival of the skin grafts. These results indicate that B-cells and/or Ig "help" T-cells through the generation and maintenance of T-cell diversity, improving T-cell function. These results, again, may have important implications on therapy and immune improvement in the context of AIDS, cancer, autoimmunity and post-myeloablative treatments, where immune reconstitution occurs.

The T cell repertoire may be influenced by the immunoglobulin idiotypic

peptides presented either by B cells or by other antigen presenting cells. The presence of polyclonal immunoglobulin or of immunoglobulin Fab fragment seems to overcome the impact of the absence of B cells in what concerns the diversity of TCR Vβ, since their administration to mice lacking B cells significantly enhance TCR Vβ diversity.

The impact of immunoglobulin idiotypic peptides on TCR cell diversification and function could be seen as a further development of Jerne's idiotypic network theory and as another potential usefulness of immunoglobulin, in addition of its recognized roles on the treatment of B cell immunodeficiencies and autoimmune, inflammatory and infectious disorders. Thus, the development of strategies that increase the diversity of the T cell repertoire may improve T cell function in those situations and others characterized by a contracted TCR repertoire, such as AIDS, cancer and following chemotherapy and hematopoietic precursors transplantation.

In addition, the experimental data obtained in animal models and described in this work give a new perspective to the way primary immunodeficiencies are generally interpreted. In the light of these new findings, the separation of B and T cell immunodeficiencies may not be adequate. In fact, a B cell immunodeficiency implicates a decrease in T cell function, through impairment upon TCR repertoire diversity. Also, a T cell immunodeficiency is known to originate an impairment of B cell function since a vast number of antigens depend upon T cells for their presentation to B cells. The present work proposes that B and T cell immunodeficiencies should be seen as

common deregulations of adaptive immunity. Also, the therapeutic approach for those situations should take into account the influence exerted by the cellular and humoral immune compartments upon each other. This could be done, for instance, through the use of polyclonal immunoglobulin, which would improve both B and T cell functions.

The clarification of the mechanism of action of immunoglobulins in various clinical settings can be extremely relevant. It should be noted that recently the infusion of polyclonal immunoglobulin as an adjuvant treatment for sepsis was re-assessed. This was a consequence of a meta-analysis including all randomized, controlled trials of patients with sepsis who received polyclonal immunoglobulin therapy or placebo or no intervention [277]. The conclusions of this meta-analysis were that a survival benefit was observed for critically ill patients with infections receiving polyclonal immunoglobulin compared to those who received placebo or no intervention. This result shows the immune modulator effect carried out by polyclonal immunoglobulin and is an example of the clinical relevance of the experimental work presented here.

Polyclonal immunoglobulin represents another bond between B and T cell compartments at the development stage and at functional level of mature cells. Also, immunoglobulin derivatives, like Fab fragments, can be used to improve T cell function *in vivo* during immune reconstitution, avoiding some immunosuppressive effects of polyclonal immunoglobulin. The *in vivo* administration of the whole molecule of immunoglobulin may have

immunosuppressive properties since Fc receptors, present in a vast number of immunological cells and having crucial functions on the activation of those cells, are blocked by the binding of the Fc portion of the immunoglobulin molecule.

The second part of the work presented in this dissertation, applies this new view of interactions within the immune system to the clinical setting. More specifically, the immune reconstitution after myeloablative treatments in onco-hematology was evaluated, based on preliminary data published by Dr. Porrata et al [82, 84, 234]. This evaluation was performed in patients with haematological tumours (lymphomas and myelomas) undergoing high dose anti-neoplastic therapy with autologous haematopoietic support. The data obtained establish that the robustness of the reconstituted immune system after autologous stem cell transplantation is a major determinant predicting progression free survival and overall survival in those patients. In particular, I examined a series of patients with mantle cell lymphoma undergoing autologous hematopoietic stem cell transplantation and another series of patients with non-Hodgkin lymphoma treated with rituximab. In the case of MCL patients, the early recovery of higher absolute lymphocytes numbers after ASCT predicts a significantly longer survival (overall and without relapse). This suggests that a robust immune system would offer an advantage in situations of immune reconstitution. When patients with NHL

were treated with rituximab, the presence of sufficient numbers of lymphocytes before the treatment, in particular of CD4+ T cells, predicted a lower risk of relapse.

Also, in agreement with the results from the experimental models showing that treatment of immunodeficient mice with polyclonal immunoglobulin was able to increase TCR repertoire diversity and to improve T cell function. I showed that, in the case of multiple myeloma patients in remission after autologous hematopoietic stem cell transplantation, the recovery of the polyclonal immunoglobulin compartment is an independent factor predicting relapse free survival. This effect was shown to be independent of other well known predictive factors in multiple myeloma such as β2 microglobulin and cytogenetic abnormalities. These data suggest that the recovery of the polyclonal compartment of immunoglobulin during immune reconstitution after autologous stem cell transplantation in patients with multiple myeloma is crucial to re-inforce the immune system, decreasing the risk of relapse. These observations set the ground for future clinical studies testing the impact of treatment of these patients with IVIg as a way of boost their immune system.

The work presented here represents, in my view, a step forward on the knowledge of T cell development, B cell-T cell interdependency and relationship, the function of immunoglobulin and its effect on T cell immunity.

Understanding the effect of immunoglobulin following autologous stem cell transplantation *in vivo* will potentially creates an opportunity to modulate this process in patients after autologous stem cell transplantation, facilitating T cell immune recovery in this setting.

Also, the use of polyclonal immunoglobulin (i.e., IVIg) in patients with B cells primary immunodeficiency, hypogammaglobulinemia or viral infections might, not only restore the serum level of IgG, decrease autoimmunity and inflammation but also improve T cell functions.

As a consequence of the results already obtained in animal models and in the setting of autologous transplantation, my areas of interests are now mainly the application of the fundamental immunological concepts studied. Two new scientific projects were started and are under development. The first is a prospective laboratorial study aiming to determine the association between the recovery of serum immunoglobulin levels and T cell immunity (characterized in terms of measurement of recent thymic emigrants, proportion of naïve versus memory CD4+ and CD8+ T cells and TCR diversity) following ASCT for non-Hodgkin's lymphoma. The project is being conducted at the Mayo Clinic, Rochester, MN, USA with Dr. Luis Porrata and Professor Svetomir Markovic and is funded by a research grant from the University of Iowa/Mayo Clinic Lymphoma SPORE Developmental Research Grants. We hypothesized that T cell immune reconstitution after ASCT may

be dependent on post-ASCT immunoglobulin recovery. Higher serum immunoglobulin levels may be associated with a more effective *de novo* T cell reconstitution, an increase in the number of recent thymic emigrants in the peripheral blood, an enlargement of the compartment of naïve T cells and an increase in T cell repertoire diversity. The study is designed to accrue 30 non-Hodgkin's lymphoma adult patients who are candidates to ASCT. Peripheral serum levels of immunoglobulin, recent thymic emigrants, the proportion of naïve versus memory CD4+ and CD8+ T cells and TCR diversity will be analysed longitudinality. We will analyze the relationship between the variables, indicating to which extent one variable can be predicted by knowing the others, including the examination of potential confounding factors (as age and baseline levels). Our goal in this study is generation of preliminary, hypothesis generating data that will be the subject of further clinical and laboratory investigations.

This study is a significant step in the field of lymphoma clinical research as it integrates basic knowledge about immunoglobulin/T cell interactions with clinical observations of post-ASCT immune reconstitution. If successful, the data may further support our argument that early immune recovery following ASCT may be one of the most important clinical goals in autologous hematopoietic stem cell transplantation.

The other project is being developed at IGC, Oeiras and started in 2006. The goal is to determine whether immunoglobulin or its derivatives, such as Fc or Fab fragments, contribute to an improved immune reconstitution after ASCT,

and to determine whether injections of immunoglobulin or fragments can improve *in vivo* T cell immune function in mice after ASCT. The T cell functions will be tested using graft rejection and, possibly, an infection model. Because diversity of the T lymphocytes is directly related to T cell function [190, 278], this work will examine whether critical defects in immunity can be repaired by increasing TCR diversity and T cell function following treatment with immunoglobulin or derivatives. This research will directly address the immune reconstitution after ASCT but may also provide clues of central interest for the fields of AIDS and aging, where immune function is an essential determinant for survival [190, 279]. The project is funded by Associação Portuguesa Contra a Leucemia and Fundação para a Ciência e Tecnologia.

Bibliography

1. **Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annual Review of Immunology, 2002. 20: p. 197-216.**
2. **Cooper, M.D. and M.N. Alder, *The evolution of adaptive immune systems.* Cell, 2006. 124(4): p. 815-22.**
3. **Burnet, M.F., *The Clonal Selection Theory of Acquired Immunity.* Cambridge University Press, Cambridge (1959). 1959.**
4. **Cooper, M.D., R.D. Peterson, and R.A. Good, *Delineation of the Thymic and Bursal Lymphoid Systems in the Chicken.* Nature, 1965. 205: p. 143-6.**
5. **Glick, B., T. Chang, and R. Jaap, *The Bursa of Fabricius and Antibody Production.* Poultry Science, 1956. 35: p. 224-225.**
6. **Miller, J.F., *Immunologic function of the thymus.* Lancet, 1961. 2: p. 748-749.**
7. **Arnason, B.G., B.D. Jankovic, and B.H. Waksman, *Effect of thymectomy on 'delayed' hypersensitive reactions.* Nature, 1962. 194: p. 99-100.**
8. **Szenberg, A. and N.L. Warner, *Dissociation of immunological responsiveness in fowls with a hormonally arrested development of lymphoid tissues.* Nature, 1962. 194: p. 146-147.**
9. **Szenberg, A. and N.L. Warner, *Immunological Reactions of Bursaless Fowls to Homograft Antigens.* Ann N Y Acad Sci, 1964. 120: p. 150-61.**
10. **Roitt, I.M., et al., *The cellular basis of immunological responses. A synthesis of some current views.* Lancet, 1969. 2(7616): p. 367-71.**
11. **Castellino, F. and R.N. Germain, *Cooperation between CD4+ and CD8+ T cells: when, where, and how.* Annual Review of Immunology, 2006. 24: p. 519-40.**
12. **Sprent, J. and C.D. Surh, *T cell memory.* Annual Review of Immunology, 2002. 20: p. 551-79.**
13. **Tan, J.T. and C.D. Surh, *T cell memory.* Current Topics in Microbiology & Immunology, 2006. 311: p. 85-115.**
14. **Sakaguchi, S., *Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses.* Annual Review of Immunology, 2004. 22: p. 531-62.**
15. **Coutinho, A., et al., *Thymic commitment of regulatory T cells is a pathway of TCR-dependent selection that isolates repertoires undergoing positive or negative selection.* Current Topics in Microbiology & Immunology, 2005. 293: p. 43-71.**
16. **Bluestone, J.A. and A.K. Abbas, *Natural versus adaptive regulatory T cells.* Nature Reviews. Immunology, 2003. 3(3): p. 253-7.**
17. **Brigl, M. and M.B. Brenner, *CD1: antigen presentation and T cell function.* Annual Review of Immunology, 2004. 22: p. 817-90.**
18. **Chien, Y.H. and Y. Konigshofer, *Antigen recognition by gammadelta T cells.* Immunological Reviews, 2007. 215: p. 46-58.**
19. **Keshavarzi, S., et al., *The possibility of B-cell-dependent T-cell development.* Scandinavian Journal of Immunology, 2003. 57(5): p. 446-452.**

20. **Spits, H.,** ***Development of alphabeta T cells in the human thymus.*** **Nature Reviews. Immunology, 2002. 2(10): p. 760-72.**
21. **Davis, M.M., et al.,** ***A murine T cell receptor gene complex: isolation, structure and rearrangement.*** **Immunological Reviews, 1984. 81: p. 235-58.**
22. **Arstila TP, C.A., Baron V et al.,** ***A direct estimate of the human ab T cell receptor diversity.*** **Science, 1999. 286: p. 958-961.**
23. **Kesmir, C., J.A.M. Borghans, and R.J. de Boer,** ***Diversity of human alpha beta T cell receptors.*** **Science, 2000. 288: p. 1135a.**
24. **Casrouge, A., et al.,** ***Size estimate of the alpha beta TCR repertoire of naive mouse splenocytes.*** **Journal of Immunology, 2000. 164(11): p. 5782-7.**
25. **Zinkernagel, R.M. and A. Althage,** ***On the role of thymic epithelium vs. bone marrow-derived cells in repertoire selection of T cells.*** **Proceedings of the National Academy of Sciences of the United States of America, 1999. 96(14): p. 8092-7.**
26. **Catherine Mazza , B.M.,** ***What guides MHC-restricted TCR recognition?*** **Semin Immunol., 2007. May 21; [Epub ahead of print].**
27. **Anderson, M.S., et al.,** ***Projection of an immunological self shadow within the thymus by the aire protein.[see comment].*** **Science, 2002. 298(5597): p. 1395-401.**
28. **Grigg, A. and D. Ritchie,** ***Graft-versus-lymphoma effects: clinical review, policy proposals, and immunobiology.*** **Biology of Blood & Marrow Transplantation, 2004. 10(9): p. 579-90.**
29. **Philip, T., et al.,** ***Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma.[see comment].*** **New England Journal of Medicine, 1995. 333(23): p. 1540-5.**
30. **Zittoun, R.A., et al.,** ***Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. European Organization for Research and Treatment of Cancer (EORTC) and the Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto (GIMEMA) Leukemia Cooperative Groups.[see comment].*** **New England Journal of Medicine, 1995. 332(4): p. 217-23.**
31. **Attal, M., et al.,** ***A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais du Myelome.[see comment].*** **New England Journal of Medicine, 1996. 335(2): p. 91-7.**
32. **Humblet, Y., et al.,** ***Late intensification chemotherapy with autologous bone marrow transplantation in selected small-cell carcinoma of the lung: a randomized study.*** **Journal of Clinical Oncology, 1987. 5(12): p. 1864-73.**
33. **Bezwoda, W.R., L. Seymour, and R.D. Dansey,** ***High-dose chemotherapy with hematopoietic rescue as primary treatment for metastatic breast cancer: a randomized trial.[see comment][retraction in J Clin Oncol. 2001 Jun 1;19(11):2973; PMID: 11387377].*** **Journal of Clinical Oncology, 1995. 13(10): p. 2483-9.**
34. ***Annual Report, Center for International Blood and Marrow Transplant Research.*** **CIBMTR, 2005.**

35. **Jaffe ES, H.N., Stein H,Vardiman JW, eds.,** ***World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues.*** **Lyon, France: IARC Press, 2001.**
36. **Johnson, P. and M. Glennie,** ***The mechanisms of action of rituximab in the elimination of tumor cells.*** **Seminars in Oncology, 2003. 30(1 Suppl 2): p. 3-8.**
37. **Society, A.C.,** ***Cancer Facts and Figures 2006.*** **Available at http://www.cancer.org.**
38. **Gertz, M.A.,** ***Relevant prognostic features of multiple myeloma and the new International Staging System.*** **Leukemia & Lymphoma, 2007. 48(3): p. 458-68.**
39. **Richardson PG, M.C., Schlossman R, Munshi N, Anderson K,** ***New drugs for myeloma.*** **Oncologist., 2007. 12(6): p. 664-89.**
40. **Cavallo, F., et al.,** ***The treatment of the elderly multiple myeloma patients.*** **Leukemia & Lymphoma, 2007. 48(3): p. 469-80.**
41. **Child, J.A., et al.,** ***High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma.[see comment].*** **New England Journal of Medicine, 2003. 348(19): p. 1875-83.**
42. **Bruno, B., et al.,** ***A comparison of allografting with autografting for newly diagnosed myeloma.[see comment].*** **New England Journal of Medicine, 2007. 356(11): p. 1110-20.**
43. **Lowenberg, B., et al.,** ***Autologous bone marrow transplantation in acute myeloid leukemia in first remission: results of a Dutch prospective study.*** **Journal of Clinical Oncology, 1990. 8(2): p. 287-94.**
44. **Klingemann, H.G. and G.L. Phillips,** ***Immunotherapy after bone marrow transplantation.*** **Bone Marrow Transplantation, 1991. 8(2): p. 73-81.**
45. **Petersen, F.B., et al.,** ***Autologous marrow transplantation for malignant lymphoma: a report of 101 cases from Seattle.*** **Journal of Clinical Oncology, 1990. 8(4): p. 638-47.**
46. **Coiffier, B., et al.,** ***CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma.[see comment].*** **New England Journal of Medicine, 2002. 346(4): p. 235-42.**
47. **Hiddemann, W., et al.,** ***Frontline therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) significantly improves the outcome for patients with advanced-stage follicular lymphoma compared with therapy with CHOP alone: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group.*** **Blood, 2005. 106(12): p. 3725-32.**
48. **Cartron, G., et al.,** ***From the bench to the bedside: ways to improve rituximab efficacy.*** **Blood, 2004. 104(9): p. 2635-42.**
49. **Anolik, J.H., et al.,** ***B cell reconstitution after rituximab treatment of lymphoma recapitulates B cell ontogeny.*** **Clin Immunol, 2007. 122(2): p. 139-45.**
50. **Nishio M, F.K., Yamamoto S, Endo T, Sakai T, Obara M, Kumano K, Yamaguchi K, Takeda Y, Goto H, Sato N, Koizumi K, Mukai M, Koike T.,** ***Delayed redistribution of CD27, CD40 and CD80 positive B cells and the impaired in vitro immunoglobulin production in patients with non-Hodgkin***

lymphoma after rituximab treatment as an adjuvant to autologous stem cell transplantation. **Br J Haematol, 2007. 137(4): p. 349-54.**

51. Ansell, S.M., et al., ***Phase 1 study of interleukin-12 in combination with rituximab in patients with B-cell non-Hodgkin lymphoma.*** **Blood, 2002. 99(1): p. 67-74.**

52. Berdeja JG, H.A., Lucas DM, O'Donnell P, Ambinder RF, Diehl LF, Carter-Brookins D, Newton S, Flinn IW., ***Systemic interleukin-2 and adoptive transfer of lymphokine-activated killer cells improves antibody-dependent cellular cytotoxicity in patients with relapsed B-cell lymphoma treated with rituximab.*** **Clin Cancer Res., 2007. 13(8): p. 2392-9.**

53. Sacchi, S., et al., ***Clinical activity and safety of combination immunotherapy with IFN-alpha 2a and Rituximab in patients with relapsed low grade non-Hodgkin's lymphoma.*** **Haematologica, 2001. 86(9): p. 951-8.**

54. Herberman, R.B., ***Effect of alpha-interferons on immune function.*** **Seminars in Oncology, 1997. 24(3 Suppl 9): p. S9-78-S9-80.**

55. Masztalerz, A., et al., ***Mechanisms of macrophage cytotoxicity in IL-2 and IL-12 mediated tumour regression.*** **Cancer Immunology, Immunotherapy, 2003. 52(4): p. 235-42.**

56. Golay, J., et al., ***Rituximab-mediated antibody-dependent cellular cytotoxicity against neoplastic B cells is stimulated strongly by interleukin-2.*** **Haematologica, 2003. 88(9): p. 1002-12.**

57. Dunne, J., et al., ***Selective expansion and partial activation of human NK cells and NK receptor-positive T cells by IL-2 and IL-15.*** **Journal of Immunology, 2001. 167(6): p. 3129-38.**

58. Langrish, C.L., et al., ***IL-12 and IL-23: master regulators of innate and adaptive immunity.*** **Immunological Reviews, 2004. 202: p. 96-105.**

59. Hernandez-Ilizaliturri, F.J., et al., ***Concurrent administration of granulocyte colony-stimulating factor or granulocyte-monocyte colony-stimulating factor enhances the biological activity of rituximab in a severe combined immunodeficiency mouse lymphoma model.*** **Leukemia & Lymphoma, 2005. 46(12): p. 1775-84.**

60. Niitsu, N., et al., ***Phase I study of Rituximab-CHOP regimen in combination with granulocyte colony-stimulating factor in patients with follicular lymphoma.*** **Clinical Cancer Research, 2004. 10(12 Pt 1): p. 4077-82.**

61. Sica, S., et al., ***Immune reconstitution following transplantation of autologous peripheral CD34+ cells.*** **Acta Haematologica, 2001. 105(3): p. 179-87.**

62. Rutella, S., et al., ***Immune reconstitution after transplantation of autologous peripheral CD34+ cells: analysis of predictive factors and comparison with unselected progenitor transplants.*** **British Journal of Haematology, 2000. 108(1): p. 105-15.**

63. Damiani, D., et al., ***CD34+-selected versus unmanipulated autologous stem cell transplantation in multiple myeloma: impact on dendritic and immune recovery and on complications due to infection.*** **Annals of Oncology, 2003. 14(3): p. 475-80.**

64. **Douek, D.C., et al.,** ***Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution.[see comment].*** **Lancet, 2000. 355(9218): p. 1875-81.**
65. **Schwinger, W., et al.,** ***Immune reconstitution after purified autologous and allogeneic blood stem cell transplantation compared with unmanipulated bone marrow transplantation in children.*** **British Journal of Haematology, 2006. 135(1): p. 76-84.**
66. **Guillaume, T., D.B. Rubinstein, and M. Symann,** ***Immune reconstitution and immunotherapy after autologous hematopoietic stem cell transplantation.*** **Blood, 1998. 92(5): p. 1471-90.**
67. **Villers, D., et al.,** ***Alteration of the T cell repertoire after bone marrow transplantation.*** **Bone Marrow Transplantation, 1994. 13(1): p. 19-26.**
68. **Mackall, C.L., et al.,** ***Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy.[see comment].*** **New England Journal of Medicine, 1995. 332(3): p. 143-9.**
69. **Suzuki, I., et al.,** ***Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: lack of somatic mutation indicates a maturational arrest.[see comment].*** **Blood, 1996. 87(5): p. 1873-80.**
70. **Avanzini, M.A., et al.,** ***B lymphocyte reconstitution after hematopoietic stem cell transplantation: functional immaturity and slow recovery of memory CD27+ B cells.*** **Exp Hematol, 2005. 33(4): p. 480-6.**
71. **Miller, R.A., et al.,** ***Clonal analysis of T-cell deficiencies in autotransplant recipients.*** **Blood, 1991. 77(8): p. 1845-50.**
72. **Machatschek, J., et al.,** ***Immune reconstitution, infectious complications and post transplant supportive care measures after autologous blood and marrow transplantation in children.*** **Bone Marrow Transplantation, 2003. 32(7): p. 687-93.**
73. **Kalwak, K., et al.,** ***Immune reconstitution after haematopoietic cell transplantation in children: immunophenotype analysis with regard to factors affecting the speed of recovery.*** **British Journal of Haematology, 2002. 118(1): p. 74-89.**
74. **Porrata, L.F., M.R. Litzow, and S.N. Markovic,** ***Immune reconstitution after autologous hematopoietic stem cell transplantation.*** **Mayo Clinic Proceedings, 2001. 76(4): p. 407-12.**
75. **Reittie, J.E., et al.,** ***Endogenously generated activated killer cells circulate after autologous and allogeneic marrow transplantation but not after chemotherapy.*** **Blood, 1989. 73(5): p. 1351-8.**
76. **Scheid, C., et al.,** ***Time-course of the recovery of cellular immune function after high-dose chemotherapy and peripheral blood progenitor cell transplantation for high-grade non-Hodgkin's lymphoma.*** **Bone Marrow Transplantation, 1995. 15(6): p. 901-6.**
77. **Silva, M.R., A. Parreira, and J.L. Ascensao,** ***Natural killer cell numbers and activity in mobilized peripheral blood stem cell grafts: conditions for in vitro expansion.*** **Experimental Hematology, 1995. 23(14): p. 1676-81.**

78. **Hanenberg, H., et al.,** ***Time course of interferon-gamma production deficiency after autologous and allogeneic stem cell transplantation for malignancies.*** **Experimental Hematology, 1995. 23(14): p. 1543-52.**
79. **Leandro, M.J., et al.,** ***Reconstitution of peripheral blood B cells after depletion with rituximab in patients with rheumatoid arthritis.*** **Arthritis Rheum, 2006. 54(2): p. 613-20.**
80. **Roll, P., et al.,** ***Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis.*** **Arthritis & Rheumatism, 2006. 54(8): p. 2377-86.**
81. **Vallerskog, T., et al.,** ***Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE.*** **Clinical Immunology, 2007. 122(1): p. 62-74.**
82. **Porrata, L.F., et al.,** ***Early lymphocyte recovery predicts superior survival after autologous hematopoietic stem cell transplantation in multiple myeloma or non-Hodgkin lymphoma.*** **Blood, 2001. 98(3): p. 579-85.**
83. **Porrata, L.F., et al.,** ***Early lymphocyte recovery predicts superior survival after autologous hematopoietic stem cell transplantation for patients with primary systemic amyloidosis.*** **Clinical Cancer Research, 2005. 11(3): p. 1210-8.**
84. **Porrata, L.F., et al.,** ***Early lymphocyte recovery post-autologous haematopoietic stem cell transplantation is associated with better survival in Hodgkin's disease.*** **British Journal of Haematology, 2002. 117(3): p. 629-33.**
85. **Porrata, L.F. and S.N. Markovic,** ***Timely reconstitution of immune competence affects clinical outcome following autologous stem cell transplantation.*** **Clinical & Experimental Medicine, 2004. 4(2): p. 78-85.**
86. **Nieto, Y., et al.,** ***Prognostic analysis of early lymphocyte recovery in patients with advanced breast cancer receiving high-dose chemotherapy with an autologous hematopoietic progenitor cell transplant.*** **Clinical Cancer Research, 2004. 10(15): p. 5076-86.**
87. **Ferrandina, G., et al.,** ***Lymphocyte recovery in advanced ovarian cancer patients after high-dose chemotherapy and peripheral blood stem cell plus growth factor support: clinical implications.*** **Clinical Cancer Research, 2003. 9(1): p. 195-200.**
88. **Janeway, C.A. and F.S. Rosen,** ***The gamma globulins. IV. Therapeutic uses of gamma globulin.*** **New England Journal of Medicine, 1966. 275(15): p. 826-31.**
89. **Knezevic-Maramica, I. and M.S. Kruskall,** ***Intravenous immune globulins: an update for clinicians.*** **Transfusion, 2003. 43(10): p. 1460-80.**
90. **Stiehm, E.R.,** ***Human intravenous immunoglobulin in primary and secondary antibody deficiencies.*** **Pediatric Infectious Disease Journal, 1997. 16(7): p. 696-707.**
91. **Kazatchkine, M.D. and S.V. Kaveri,** ***Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin.*** **New England Journal of Medicine, 2001. 345(10): p. 747-55.**
92. **Yu, Z. and V.A. Lennon,** ***Mechanism of intravenous immune globulin therapy in antibody-mediated autoimmune diseases.[see comment].*** **New England Journal of Medicine, 1999. 340(3): p. 227-8.**

93. **Hemming, V.,** ***Use of intravenous immunoglobulins for prophylaxis or treatment of infectious diseases.*** **Clin Diagn Lab Immunol, 2001. 8: p. 859-863.**
94. **Bayry, J., et al.,** ***Intravenous immunoglobulin for infectious diseases: back to the pre-antibiotic and passive prophylaxis era?*** **Trends in Pharmacological Sciences, 2004. 25(6): p. 306-10.**
95. **Pildal, J. and P.C. Gotzsche,** ***Polyclonal immunoglobulin for treatment of bacterial sepsis: a systematic review.*** **Clinical Infectious Diseases, 2004. 39(1): p. 38-46.**
96. **Omwandho, C.O., et al.,** ***Intravenous immunoglobulin (IVIG): modes of action in the clinical management of recurrent pregnancy loss (RPL) and selected autoimmune disorders.*** **Clinical Chemistry & Laboratory Medicine, 2004. 42(4): p. 359-70.**
97. **Samuelsson, A., T.L. Towers, and J.V. Ravetch,** ***Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor.[see comment].*** **Science, 2001. 291(5503): p. 484-6.**
98. **Negi V-S, E.S., Siberil S, Dubois-Graff S, Mouthon L, Kazatchkine, Desmazes-Lacroix S, Bayry J, Kaveri V S,** ***Intravenous Immunoglobulin: an update on the clinical use and mechanisms of action.*** **Journal of Clinical Immunology, 2007. 27(3): p. 233-245.**
99. **Kurt-Jones, E.A., et al.,** ***The role of antigen-presenting B cells in T cell priming in vivo. Studies of B cell-deficient mice.*** **Journal of Immunology, 1988. 140(11): p. 3773-8.**
100. **Constant, S.L.,** ***B lymphocytes as antigen-presenting cells for CD4+ T cell priming in vivo.*** **Journal of Immunology, 1999. 162(10): p. 5695-703.**
101. **Weissberger, H.Z., R.R. Shenk, and H.B. Dickler,** ***Antiidiotype stimulation of antigen-specific antigen-independent antibody responses in vitro. I. Evidence for stimulation of helper T lymphocyte function.*** **J Exp Med, 1983. 158(2): p. 465-76.**
102. **Ptak, W., C.A. Janeway, Jr., and P.M. Flood,** ***Immunoregulatory role of Ig isotypes. II. Activation of cells that block induction of contact sensitivity responses by antibodies of IgG2a and IgG2b isotypes.*** **J Immunol, 1988. 141(3): p. 765-73.**
103. **Fu, Y.X., et al.,** ***B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin alpha-dependent fashion.*** **J Exp Med, 1998. 187(7): p. 1009-18.**
104. **Olson, T.S., et al.,** ***Expanded B cell population blocks regulatory T cells and exacerbates ileitis in a murine model of Crohn disease.*** **J Clin Invest, 2004. 114(3): p. 389-98.**
105. **Ansel, K.M., et al.,** ***A chemokine-driven positive feedback loop organizes lymphoid follicles.*** **Nature, 2000. 406(6793): p. 309-314.**
106. **Cyster, J.G.,** ***B cells on the front line.*** **Nature Immunology, 2000. 1(1): p. 9-10.**
107. **Cyster, J.G., et al.,** ***Follicular stromal cells and lymphocyte homing to follicles.*** **Immunological Reviews, 2000. 176: p. 181-193.**
108. **Longo, N.S. and P.E. Lipsky,** ***Somatic hypermutation in human B cell subsets.*** **Springer Semin Immunopathol, 2001. 23(4): p. 367-85.**

109. Golovkina, T.V., et al., ***Organogenic role of B lymphocytes in mucosal immunity.*** **Science, 1999. 286(5446): p. 1965-1968.**
110. Lund, F.E., et al., ***B cells are required for generation of protective effector and memory CD4 cells in response to Pneumocystis lung infection.*** **Journal of Immunology, 2006. 176(10): p. 6147-54.**
111. Marrack, P., et al., ***T-cell survival.*** **Immunological Reviews, 1998. 165: p. 279-85.**
112. Viret, C., F.S. Wong, and C.A. Janeway, Jr., ***Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition.*** **Immunity, 1999. 10(5): p. 559-68.**
113. Neuberger, M.S., ***Antigen receptor signaling gives lymphocytes a long life.[comment].*** **Cell, 1997. 90(6): p. 971-3.**
114. Linton, P., J. Harbertson, and L.M. Bradley, ***A critical role for B cells in the development of memory CD4 cells.*** **Journal of Immunology, 2000. 165: p. 5558-5565.**
115. van Essen, D., et al., ***Cellular interactions involved in Th cell memory.*** **Journal of Immunology, 2000. 165: p. 3640-3646.**
116. van Essen, D., P. Dullforce, and D. Gray, ***Role of B cells in maintaining helper T-cell memory.*** **Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 2000. 355(1395): p. 351-355.**
117. Pannetier, C., et al., ***The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments.*** **Proceedings of the National Academy of Sciences of the United States of America, 1993. 90(9): p. 4319-23.**
118. Pannetier, C., J. Even, and P. Kourilsky, ***T-cell repertoire diversity and clonal expansions in normal and clinical samples.*** **Immunology Today, 1995. 16(4): p. 176-81.**
119. Gorochov, G., et al., ***Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy.*** **Nat Med, 1998. 4(2): p. 215-21.**
120. Collette, A. and A. Six, ***ISEApeaks: an Excel platform for GeneScan and Immunoscope data retrieval, management and analysis.*** **Bioinformatics, 2002. 18(2): p. 329-30.**
121. Collette, A., et al., ***New methods and software tools for high throughput CDR3 spectratyping. Application to T lymphocyte repertoire modifications during experimental malaria.*** **Journal of Immunological Methods, 2003. 278(1-2): p. 105-16.**
122. Murata, H., et al., ***T cell receptor repertoire of T cells in the kidneys of patients with lupus nephritis.*** **Arthritis & Rheumatism, 2002. 46(8): p. 2141-7.**
123. Baum, P.D. and J.M. McCune, ***Direct measurement of T-cell receptor repertoire diversity with AmpliCot.[see comment].*** **Nature Methods, 2006. 3(11): p. 895-901.**
124. Wagner, U.G., et al., ***Perturbation of the T cell repertoire in rheumatoid arthritis.*** **Proceedings of the National Academy of Sciences of the United States of America, 1998. 95(24): p. 14447-52.**

125. **Langerak, A.W., et al.,** ***Molecular and flow cytometric analysis of the Vbeta repertoire for clonality assessment in mature TCRalphabeta T-cell proliferations.*** **Blood, 2001. 98(1): p. 165-73.**
126. **Fujishima M, H.M., Fujishima N, Sawada K,** ***TCRalphabeta repertoire diversity of human naturally occuring CD4+ CD25+ regulatory T cells.*** **Immunological Letters, 2005. 99(2): p. 193-197.**
127. **Sheehan, K.M. and P.H. Brodeur,** ***Molecular cloning of the primary IgH repertoire: a quantitative analysis of VH gene usage in adult mice.*** **EMBO Journal, 1989. 8(8): p. 2313-20.**
128. **Wack, A., et al.,** ***An improved PCR-heteroduplex method permits high-sensitivity detection of clonal expansions in complex T cell populations.*** **Journal of Immunological Methods, 1996. 196(2): p. 181-92.**
129. **Cascalho, M., et al.,** ***A quasi-monoclonal mouse.[see comment].*** **Science, 1996. 272(5268): p. 1649-52.**
130. **Cascalho, M., J. Wong, and M. Wabl,** ***VH gene replacement in hyperselected B cells of the quasimonoclonal mouse.*** **Journal of Immunology, 1997. 159(12): p. 5795-801.**
131. **Chen, J., et al.,** ***Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the JH locus.*** **International Immunology, 1993. 5(6): p. 647-56.**
132. **Lopez-Macias, C., et al.,** ***Secondary rearrangements and hypermutation generate sufficient B cell diversity to mount protective antiviral immunoglobulin responses.*** **Journal of Experimental Medicine, 1999. 189(11): p. 1791-8.**
133. **West, L.J., et al.,** ***ABO-incompatible heart transplantation in infants.[see comment].*** **New England Journal of Medicine, 2001. 344(11): p. 793-800.**
134. **Correia-Neves, M., et al.,** ***The shaping of the T cell repertoire.*** **Immunity, 2001. 14(1): p. 21-32.**
135. **Saubermann, L.J., et al.,** ***Evidence of T cell receptor beta-chain patterns in inflammatory and noninflammatory bowel disease states.*** **American Journal of Physiology, 1999. 276(3 Pt 1): p. G613-21.**
136. **Mizoguchi, A., et al.,** ***Limited CD4 T-cell diversity associated with colitis in T-cell receptor alpha mutant mice requires a T helper 2 environment.*** **Gastroenterology, 2000. 119(4): p. 983-95.**
137. **Peggs, K.S., et al.,** ***Assessing diversity: immune reconstitution and T-cell receptor BV spectratype analysis following stem cell transplantation.*** **British Journal of Haematology, 2003. 120(1): p. 154-65.**
138. **Natarajan, V., et al.,** ***Increased peripheral expansion of naive CD4+ T cells in vivo after IL-2 treatment of patients with HIV infection.*** **Proceedings of the National Academy of Sciences of the United States of America, 2002. 99(16): p. 10712-7.**
139. **Farci, P., et al.,** ***The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies.*** **Science, 2000. 288(5464): p. 339-44.**
140. **Goldrath, A.W. and M.J. Bevan,** ***Selecting and maintaining a diverse T-cell repertoire.*** **Nature, 1999. 402: p. 255-262.**

141. **von Boehmer, H., *Developmental biology of T cells in T cell-receptor transgenic mice.* Annual Review of Immunology, 1990. 8: p. 531-56.**
142. **Benoist, C. and D. Mathis, *Positive selection of the T cell repertoire: where and when does it occur?* Cell, 1989. 58(6): p. 1027-33.**
143. **Pawlowski, T., et al., *Positive selection of T lymphocytes on fibroblasts.* Nature, 1993. 364(6438): p. 642-5.**
144. **Hugo, P., et al., *Fibroblasts can induce thymocyte positive selection in vivo.* Proceedings of the National Academy of Sciences of the United States of America, 1993. 90(21): p. 10335-9.**
145. **Bix, M. and D. Raulet, *Inefficient positive selection of T cells directed by haematopoietic cells.* Nature, 1992. 359(6393): p. 330-3.**
146. **Auchincloss, H., Jr., et al., *The role of "indirect" recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice.* Proc Natl Acad Sci U S A, 1993. 90(8): p. 3373-7.**
147. **Martinic, M.M., et al., *Efficient T cell repertoire selection in tetraparental chimeric mice independent of thymic epithelial MHC.* Proceedings of the National Academy of Sciences of the United States of America, 2003. 100(4): p. 1861-6.**
148. **Farr, A.G., J.L. Dooley, and M. Erickson, *Organization of thymic medullary epithelial heterogeneity: implications for mechanisms of epithelial differentiation.* Immunological Reviews, 2002. 189: p. 20-7.**
149. **Surh, C.D., et al., *Thymic selection by a single MHC/peptide ligand produces a semidiverse repertoire of CD4+ T cells.* Immunity, 1997. 7(2): p. 209-19.**
150. **Sant'Angelo, D.B., et al., *The imprint of intrathymic self-peptides on the mature T cell receptor repertoire.* Immunity, 1997. 7(4): p. 517-24.**
151. **Barton, G.M. and A.Y. Rudensky, *Requirement for diverse, low-abundance peptides in positive selection of T cells.* Science, 1999. 283(5398): p. 67-70.**
152. **Miyama-Inaba, M., et al., *Unusual phenotype of B cells in the thymus of normal mice.* Journal of Experimental Medicine, 1988. 168(2): p. 811-816.**
153. **Weiss, S. and B. Bogen, *B-lymphoma cells process and present their endogenous immunoglobulin to major histocompatibility complex-restricted T cells.* Proc Natl Acad Sci U S A, 1989. 86(1): p. 282-6.**
154. **Lanzavecchia, A., *Antigen-specific interaction between T and B cells.* Nature, 1985. 314(6011): p. 537-9.**
155. **Kitamura, D., et al., *A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene.* Nature, 1991. 350(6317): p. 423-6.**
156. **Macpherson, A.J., et al., *IgA responses in the intestinal mucosa against pathogenic and non- pathogenic microorganisms.* Microbes Infect, 2001. 3(12): p. 1021-35.**
157. **Hasan, M., et al., *Incomplete block of B cell development and immunoglobulin production in mice carrying the muMT mutation on the BALB/c background.* European Journal of Immunology, 2002. 32(12): p. 3463-71.**
158. **Surh, C.D. and J. Sprent, *T-cell apoptosis detected in situ during positive and negative selection in the thymus.* Nature, 1994. 372(6501): p. 100-3.**

159. Tough, D.F. and J. Sprent, *Measurement of T and B cell turnover with bromodeoxyuridine.* Current Protocols in Immunology, 1996. Supplement 18: p. 4.7.1-4.7.6.
160. Sprent, J. and D.F. Tough, *Lymphocyte life-span and memory.* Science, 1994. 265(5177): p. 1395-400.
161. Bradley, L.M., et al., *Availability of antigen-presenting cells can determine the extent of CD4 effector expansion and priming for secretion of Th2 cytokines in vivo.* European Journal of Immunology, 2002. 32(8): p. 2338-46.
162. Ogle, B.M., et al., *Direct measurement of lymphocyte receptor diversity.* Nucleic Acids Research, 2003. 31(22): p. e139.
163. Hangaishi, A., et al., *Mutations and loss of expression of a mismatch repair gene, hMLH1, in leukemia and lymphoma cell lines.* Blood, 1997. 89(5): p. 1740-7.
164. Akashi, K., et al., *B lymphopoiesis in the thymus.* Journal of Immunology, 2000. 164(10): p. 5221-5226.
165. Aruffo, A., et al., *The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome.* Cell, 1993. 72(2): p. 291-300.
166. Gill, J., et al., *Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells.* Nature Immunology, 2002. 3(7): p. 635-42.
167. Zinkernagel, R.M., et al., *Restriction specificities, alloreactivity, and allotolerance expressed by T cells from nude mice reconstituted with H-2-compatible or -incompatible thymus grafts.* Journal of Experimental Medicine, 1980. 151(2): p. 376-99.
168. Lopez-Macias, C., et al., *Secondary rearrangements and hypermutation generate sufficient B cell diversity to mount protective antiviral immunoglobulin responses.* Journal of Experimental Medicine, 1999. 189(11): p. 1791-1798.
169. Mazda, O., et al., *Requirement of dendritic cells and B cells in the clonal deletion of Mls-reactive T cells in the thymus.* Journal of Experimental Medicine, 1991. 173: p. 539-547.
170. Kleindienst, P., et al., *Functional comparison of thymic B cells and dendritic cells in vivo.* Blood, 2000. 95(8): p. 2610-2616.
171. Tao, Y., et al., *AIRE recruits multiple transcriptional components to specific genomic regions through tethering to nuclear matrix.* Molecular Immunology, 2006. 43(4): p. 335-45.
172. Anderson, M.S., et al., *The cellular mechanism of Aire control of T cell tolerance.[see comment].* Immunity, 2005. 23(2): p. 227-39.
173. Claverie, J.M., A. Prochnicka-Chalufour, and L. Bougueleret, *Implications of a Fab-like structure for the T-cell receptor.* Immunology Today, 1989. 10(1): p. 10-4.
174. Kaymaz, H., et al., *Reactions of anti-immunoglobulin sera with synthetic T cell receptor peptides: implications for the three-dimensional structure and function of the TCR beta chain.* International Immunology, 1993. 5(5): p. 491-502.
175. Khoruts, A. and J.M. Fraser, *A causal link between lymphopenia and autoimmunity.* Immunology Letters, 2005. 98(1): p. 23-31.

176. **Miller, J.F.A.P.,** ***Effect of neonatal thymectomy on the immunological responsiveness of the mouse.*** **Proc. Royal Soc. London, 1962. 156: p. 415-428.**
177. **Miller, J.F.,** ***Effect of thymectomy in adult mice on immunological responsiveness.*** **Nature, 1965. 208(17): p. 1337-8.**
178. **Claman, H.N., E.A. Chaperon, and R.F. Triplett,** ***Immunocompetence of transferred thymus-marrow cell combinations.*** **J Immunol, 1966. 97(6): p. 828-32.**
179. **Mastroeni, P., et al.,** ***Igh-6$^{-/-}$ (B-cell-deficient) mice fail to mount solid acquired resistance to oral challenge with virulent Salmonella enterica serovar typhimurium and show impaired Th1 T-cell responses to Salmonella antigens.*** **Infect Immun, 2000. 68(1): p. 46-53.**
180. **Elkins, K.L., C.M. Bosio, and T.R. Rhinehart-Jones,** ***Importance of B cells, but not specific antibodies, in primary and secondary protective immunity to the intracellular bacterium Francisella tularensis live vaccine strain.*** **Infect Immun, 1999. 67(11): p. 6002-7.**
181. **Langhorne, J., et al.,** ***A role for B cells in the development of T cell helper function in a malaria infection in mice.*** **Proceedings of the National Academy of Sciences of the United States of America, 1998. 95(4): p. 1730-1734.**
182. **Yang, X. and R.C. Brunham,** ***Gene knockout B cell-deficient mice demonstrate that B cells play an important role in the initiation of T cell responses to Chlamydia trachomatis (mouse pneumonitis) lung infection.*** **J Immunol, 1998. 161(3): p. 1439-46.**
183. **Wilfert, C.M., et al.,** ***Persistent and fatal central-nervous-system echovirus infections in patients with agammaglobulinemia.*** **New England Journal of Medicine, 1977. 296(26): p. 1485-1489.**
184. **Bertini, M., et al.,** ***Mantle cell lymphoma: a retrospective study on 27 patients. Clinical features and natural history.*** **Haematologica, 1998. 83(4): p. 312-6.**
185. **João, C.M., et al.,** ***B cell-dependent TCR diversification.*** **Journal of Immunology, 2004. 172(8): p. 4709-4716.**
186. **Billingham, R.E., L. Brent, and P.B. Medawar,** ***Activity acquired tolerance of foreign cells.*** **Nature, 1953. 172(4379): p. 603-6.**
187. **Cascalho, M., et al.,** ***A quasi-monoclonal mouse.*** **Science, 1996. 272(5268): p. 1649-1652.**
188. **Malissen, M., et al.,** ***Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the beta polypeptide.*** **Cell, 1984. 37(3): p. 1101-10.**
189. **Tanchot, C., et al.,** ***Differential requirements for survival and proliferation of CD8 naive or memory T cells.*** **Science, 1997. 276(5321): p. 2057-2062.**
190. **Mackall, C.L. and R.E. Gress,** ***Thymic aging and T-cell regeneration.*** **Immunological Reviews, 1997. 160: p. 91-102.**
191. **Berard, M. and D.F. Tough,** ***Qualitative differences between naive and memory T cells.*** **Immunology, 2002. 106(2): p. 127-138.**
192. **Suchin, E.J., et al.,** ***Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question.*** **Journal of Immunology, 2001. 166(2): p. 973-81.**

193. Lindahl, K.F. and D.B. Wilson, *Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors.* J Exp Med, 1977. 145(3): p. 508-22.
194. Kanagawa, O., J. Louis, and J.C. Cerottini, *Frequency and cross-reactivity od cytolytic T lymphocyte precursors reacting against male alloantigens.* Journal of Immunology, 1982. 128(5): p. 2362-6.
195. Sakaguchi, S., et al., *Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance.* Immunol Rev, 2001. 182: p. 18-32.
196. Samuelsson, A., T.L. Towers, and J.V. Ravetc, *Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor.[see comment.* Science, 2001. 291(5503): p. 484-6.
197. Eysteinsdottir, J.H., et al., *The influence of partial or total thymectomy during open heart surgery in infants on the immune function later in life.* Clinical & Experimental Immunology, 2004. 136(2): p. 349-55.
198. Halnon, N.J., et al., *Thymic function and impaired maintenance of peripheral T cell populations in children with congenital heart disease and surgical thymectomy.* Pediatric Research, 2005. 57(1): p. 42-8.
199. Sundblad, A., et al., *Normal serum immunoglobulins influence the numbers of bone marrow pre-B and B cells.* Eur J Immunol, 1991. 21(5): p. 1155-61.
200. Dietrich, G., et al., *Selection of the expressed B cell repertoire by infusion of normal immunoglobulin G in a patient with autoimmune thyroiditis.* Eur J Immunol, 1993. 23(11): p. 2945-50.
201. Freitas, A.A. and B. Rocha, *Lymphocyte survival: a red queen hypothesis.* Science, 1997. 277(5334): p. 1950.
202. Benoist, C. and D. Mathis, *Selection for survival?* Science, 1997. 276(5321): p. 2000-1.
203. Swain, S.L., *Generation and in vivo persistence of polarized Th1 and Th2 memory cells.* Immunity, 1994. 1(7): p. 543-52.
204. Murali-Krishna, K., et al., *Persistence of memory CD8 T cells in MHC class I-deficient mice.* Science, 1999. 286(5443): p. 1377-1381.
205. Swain, S.L., H. Hu, and G. Huston, *Class II-independent generation of CD4 memory T cells from effectors.* Science, 1999. 286(5443): p. 1381-1383.
206. Weisenburger, D.D. and J.O. Armitage, *Mantle cell lymphoma-- an entity comes of age.* Blood, 1996. 87(11): p. 4483-94.
207. Velders, G.A., et al., *Mantle-cell lymphoma: a population-based clinical study.* J Clin Oncol, 1996. 14(4): p. 1269-74.
208. Hiddemann, W., et al., *Mantle-cell lymphomas have more widespread disease and a slower response to chemotherapy compared with follicle-center lymphomas: results of a prospective comparative analysis of the German Low-Grade Lymphoma Study Group.* Journal of Clinical Oncology, 1998. 16(5): p. 1922-30.
209. Samaha, H., et al., *Mantle cell lymphoma: a retrospective study of 121 cases.* Leukemia, 1998. 12(8): p. 1281-7.
210. Vose, J.M., et al., *Autologous hematopoietic stem cell transplantation for mantle cell lymphoma.* Biol Blood Marrow Transplant, 2000. 6(6): p. 640-5.

211. **Decaudin, D., et al.,** ***Efficacy of autologous stem cell transplantation in mantle cell lymphoma: a 3-year follow-up study.*** **Bone Marrow Transplantation, 2000. 25(3): p. 251-6.**

212. **Dreyling, M., et al.,** ***Early consolidation by myeloablative radiochemotherapy followed by autologous stem cell transplantation in first remission significantly prolongs progression-free survival in mantle-cell lymphoma: results of a prospective randomized trial of the European MCL Network.*** **Blood, 2005. 105(7): p. 2677-84.**

213. **Oinonen, R., et al.,** ***Autologous stem cell transplantation in patients with mantle cell lymphoma.*** **Leuk Lymphoma, 2002. 43(6): p. 1229-37.**

214. **Milpied, N., et al.,** ***High-dose therapy with stem cell transplantation for mantle cell lymphoma: results and prognostic factors, a single center experience.*** **Bone Marrow Transplant, 1998. 22(7): p. 645-50.**

215. **Khouri, I.F., et al.,** ***Long-term follow-up of autologous stem cell transplantation in patients with diffuse mantle cell lymphoma in first disease remission: the prognostic value of beta2-microglobulin and the tumor score.*** **Cancer, 2003. 98(12): p. 2630-5.**

216. **Gordan, L.N., et al.,** ***Correlation of early lymphocyte recovery and progression-free survival after autologous stem-cell transplant in patients with Hodgkin's and non-Hodgkin's Lymphoma.*** **Bone Marrow Transplantation, 2003. 31(11): p. 1009-13.**

217. **Kim, H., et al.,** ***Lymphocyte recovery as a positive predictor of prolonged survival after autologous peripheral blood stem cell transplantation in T-cell non-Hodgkin's lymphoma.*** **Bone Marrow Transplantation, 2004. 34(1): p. 43-9.**

218. **Porrata, L.F., et al.,** ***Early lymphocyte recovery is a predictive factor for prolonged survival after autologous hematopoietic stem cell transplantation for acute myelogenous leukemia.*** **Leukemia, 2002. 16(7): p. 1311-8.**

219. **Porrata, L.F., et al.,** ***Prolonged survival associated with early lymphocyte recovery after autologous hematopoietic stem cell transplantation for patients with metastatic breast cancer.[see comment].*** **Bone Marrow Transplantation, 2001. 28(9): p. 865-71.**

220. ***A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project.*** **New England Journal of Medicine, 1993. 329(14): p. 987-94.**

221. **Kaplan E, M.P.,** ***Nonparametric estimation from incomplete observations.*** **J Am Stat Assoc., 1958. 53: p. 457-481.**

222. **Cox, D.,** ***Regression models and life-tables.*** **J R Stat Soc (B). 1972. 34: p. 187-202.**

223. **Harrell, F.E., Jr., et al.,** ***Evaluating the yield of medical tests.*** **JAMA, 1982. 247(18): p. 2543-6.**

224. **Brugger, W., et al.,** ***Rituximab consolidation after high-dose chemotherapy and autologous blood stem cell transplantation in follicular and mantle cell lymphoma: a prospective, multicenter phase II study.*** **Annals of Oncology, 2004. 15(11): p. 1691-8.**

225. Mangel, J., et al., ***Intensive chemotherapy and autologous stem-cell transplantation plus rituximab is superior to conventional chemotherapy for newly diagnosed advanced stage mantle-cell lymphoma: a matched pair analysis.*** **Annals of Oncology, 2004. 15(2): p. 283-90.**

226. Barnes, D.W. and J.F. Loutit, ***Treatment of murine leukaemia with x-rays and homologous bone marrow. II.*** **Br J Haematol, 1957. 3(3): p. 241-52.**

227. Horowitz, M.M., et al., ***Graft-versus-leukemia reactions after bone marrow transplantation.*** **Blood, 1990. 75(3): p. 555-62.**

228. Kolb, H.J., et al., ***Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients.[see comment].*** **Blood, 1995. 86(5): p. 2041-50.**

229. Perreault, C., et al., ***Critical issues in bone marrow transplantation immunology.*** **Bone Marrow Transplantation, 1991. 7 Suppl 1: p. 24-5.**

230. Truitt, R.L. and A.A. Atasoylu, ***Contribution of CD4+ and CD8+ T cells to graft-versus-host disease and graft-versus-leukemia reactivity after transplantation of MHC-compatible bone marrow.*** **Bone Marrow Transplantation, 1991. 8(1): p. 51-8.**

231. Pion, S., et al., ***Immunodominant minor histocompatibility antigens expressed by mouse leukemic cells can serve as effective targets for T cell immunotherapy.*** **Journal of Clinical Investigation, 1995. 95(4): p. 1561-8.**

232. Joao, C., et al., ***B cell-dependent TCR diversification.*** **Journal of Immunology, 2004. 172(8): p. 4709-16.**

233. Porrata, L.F., et al., ***The dose of infused lymphocytes in the autograft directly correlates with clinical outcome after autologous peripheral blood hematopoietic stem cell transplantation in multiple myeloma.*** **Leukemia, 2004. 18(6): p. 1085-92.**

234. Porrata, L.F., et al., ***Re-infused autologous graft natural killer cells correlates with absolute lymphocyte count recovery after autologous stem cell transplantation.*** **Leukemia & Lymphoma, 2003. 44(6): p. 997-1000.**

235. Dispenzieri, A. and R.A. Kyle, ***Multiple myeloma: clinical features and indications for therapy.*** **Bailliere's Best Practice in Clinical Haematology, 2005. 18(4): p. 553-68.**

236. Hari, P., M.C. Pasquini, and D.H. Vesole, ***Cure of multiple myeloma -- more hype, less reality.*** **Bone Marrow Transplantation, 2006. 37(1): p. 1-18.**

237. Fermand, J.P., et al., ***High-dose therapy and autologous blood stem-cell transplantation compared with conventional treatment in myeloma patients aged 55 to 65 years: long-term results of a randomized control trial from the Group Myelome-Autogreffe.*** **Journal of Clinical Oncology, 2005. 23(36): p. 9227-33.**

238. Blade, J., et al., ***High-dose therapy intensification compared with continued standard chemotherapy in multiple myeloma patients responding to the initial chemotherapy: long-term results from a prospective randomized trial from the Spanish cooperative group PETHEMA.*** **Blood, 2005. 106(12): p. 3755-9.**

239. Barlogie, B., et al., ***Long-term outcome results of the first tandem autotransplant trial for multiple myeloma.*** **British Journal of Haematology, 2006. 135(2): p. 158-64.**

240. **Jagannath, S.,** ***Treatment of myeloma in patients not eligible for transplantation.*** **Current Treatment Options in Oncology, 2005. 6(3): p. 241-53.**

241. **Sara Prutchi-Sagiv, N.G., Howard S. Oster, Odelia Katz, Amos Cohen, Elizabeth Naparstek, Moshe Mittelman,** ***Erythropoietin treatment in advanced multiple myeloma is associated with improved immunological functions: could it be beneficial in early disease?*** **British Journal of Haematology, 2006. 135(5): p. 660–672.**

242. **Munshi, N.C.,** ***Immunoregulatory mechanisms in multiple myeloma.*** **Hematology - Oncology Clinics of North America, 1997. 11(1): p. 51-69.**

243. **San Miguel, J.F., et al.,** ***Immunophenotype and DNA cell content in multiple myeloma.*** **Baillieres Clinical Haematology, 1995. 8(4): p. 735-59.**

244. **Koike, M., et al.,** ***Relationship between CD4(+)/CD8(+) T cell ratio and T cell activation in multiple myeloma: reference to IL-16.*** **Leukemia Research, 2002. 26(8): p. 705-11.**

245. **Ozer, H., et al.,** ***Immunoregulatory T cell function in multiple myeloma.*** **Journal of Clinical Investigation, 1981. 67(3): p. 779-89.**

246. **Pilarski, L.M., et al.,** ***Abnormal clonogenic potential of T cells from multiple myeloma patients.*** **Blood, 1985. 66(6): p. 1266-71.**

247. **Pilarski, L.M., B.A. Ruether, and M.J. Mant,** ***Abnormal function of B lymphocytes from peripheral blood of multiple myeloma patients. Lack of correlation between the number of cells potentially able to secrete immunoglobulin M and serum immunoglobulin M levels.*** **Journal of Clinical Investigation, 1985. 75(6): p. 2024-9.**

248. **Lahat, N., E. Aghai, and P. Froom,** ***T-cells of multiple myeloma patients triggered by the autologous mixed lymphocyte reaction suppress polyclonal immunoglobulin synthesis.*** **Cancer, 1988. 62(6): p. 1124-8.**

249. **Van den Hove, L.E., et al.,** ***Peripheral blood lymphocyte subset shifts in patients with untreated hematological tumors: evidence for systemic activation of the T cell compartment.*** **Leukemia Research, 1998. 22(2): p. 175-84.**

250. **Ullrich, S. and S. Zolla-Pazner,** ***Immunoregulatory circuits in myeloma.*** **Clinics in Haematology, 1982. 11(1): p. 87-111.**

251. **Joao, C., Porrata L.F., Inwards D.J., Ansell S.M., Micallef I.N., Johnston P.B., Gastineau D.A., Markovic S.N.,** ***Early lymphocyte recovery after autologous stem cell transplantation predicts survival in mantle-cell lymphoma.*** **Bone Marrow Transplant, 2006. 37: p. 865-871.**

252. **Joao, C., B.M. Ogle, and S. Geyer,** ***Immunoglobulin promotes the diversity and the function of T cells.*** **Eur J Immunol, 2006. 36: p. 1718-1728.**

253. **Joao, C.,** ***Immunoglobulin is a highly diverse self-molecule that improves cellular diversity and function during immune reconstitution.*** **Medical Hypotheses, 2007. 68(1): p. 158-61.**

254. **Blade, J., et al.,** ***Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant.*** **British Journal of Haematology, 1998. 102(5): p. 1115-23.**

255. **Twomey, J.J.,** ***Infections complicating multiple myeloma and chronic lymphocytic leukemia.*** **Archives of Internal Medicine, 1973. 132(4): p. 562-5.**
256. **Perri, R.T., R.P. Hebbel, and M.M. Oken,** ***Influence of treatment and response status on infection risk in multiple myeloma.*** **Am J Med, 1981. 71(6): p. 935-40.**
257. **Perez-Andres, M., et al.,** ***Characterization of bone marrow T cells in monoclonal gammopathy of undetermined significance, multiple myeloma, and plasma cell leukemia demonstrates increased infiltration by cytotoxic/Th1 T cells demonstrating a squed TCR-Vbeta repertoire.*** **Cancer, 2006. 106(6): p. 1296-305.**
258. **Hargreaves, R.M., et al.,** ***Immunological factors and risk of infection in plateau phase myeloma.*** **Journal of Clinical Pathology, 1995. 48(3): p. 260-6.**
259. **Schutt, P., et al.,** ***Immune parameters in multiple myeloma patients: influence of treatment and correlation with opportunistic infections.*** **Leukemia & Lymphoma, 2006. 47(8): p. 1570-82.**
260. **Chapel, H.M., et al.,** ***Randomised trial of intravenous immunoglobulin as prophylaxis against infection in plateau-phase multiple myeloma. The UK Group for Immunoglobulin Replacement Therapy in Multiple Myeloma.[see comment].*** **Lancet, 1994. 343(8905): p. 1059-63.**
261. **Zhao, X., et al.,** ***Comparison of ancillary studies in the detection of residual disease in plasma cell myeloma in bone marrow.*** **American Journal of Clinical Pathology, 2006. 125(6): p. 895-904.**
262. **Kay, N.E., et al.,** ***Blood levels of immune cells predict survival in myeloma patients: results of an Eastern Cooperative Oncology Group phase 3 trial for newly diagnosed multiple myeloma patients.*** **Blood, 2001. 98(1): p. 23-8.**
263. **Annette Höfer, A.B., Jens Klaus, Iris Breitkreutz, Doris Herrmann, Friedrich W. Cremer, Sabine Gerull, Thomas Moehler, Gerlinde Egerer, Ute Hegenbart, Anthony D. Ho, and Hartmut Goldschmidt,** ***Recovery of Polyclonal Immunoglobulin Synthesis after Autologous Stem Cell Transplantation in Multiple Myeloma Patients as a Prognostic Factor for Event-Free Survival.*** **Blood (ASH Annual Meeting Abstracts), 2004. 104: p.: abstract # 939.**
264. **Golay, J., et al.,** ***Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement-mediated cell lysis.*** **Blood, 2000. 95(12): p. 3900-8.**
265. **Flieger, D., et al.,** ***Mechanism of cytotoxicity induced by chimeric mouse human monoclonal antibody IDEC-C2B8 in CD20-expressing lymphoma cell lines.*** **Cellular Immunology, 2000. 204(1): p. 55-63.**
266. **Shan, D., J.A. Ledbetter, and O.W. Press,** ***Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies.*** **Blood, 1998. 91(5): p. 1644-52.**
267. **Jazirehi, A.R., et al.,** ***Rituximab (chimeric anti-CD20 monoclonal antibody) inhibits the constitutive nuclear factor-{kappa}B signaling pathway in non-Hodgkin's lymphoma B-cell lines: role in sensitization to chemotherapeutic drug-induced apoptosis.*** **Cancer Research, 2005. 65(1): p. 264-76.**

268. **Kobayashi, M., et al.,** ***Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes.*** **Journal of Experimental Medicine, 1989. 170(3): p. 827-45.**

269. **Stern, A.S., et al.,** ***Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells.*** **Proceedings of the National Academy of Sciences of the United States of America, 1990. 87(17): p. 6808-12.**

270. **Rook, A.H., et al.,** ***Interleukin-12 therapy of cutaneous T-cell lymphoma induces lesion regression and cytotoxic T-cell responses.*** **Blood, 1999. 94(3): p. 902-8.**

271. **Younes A., R.M.J., Flinn I.W., Romaguera J., Hagemeister F.B., Cabanillas F., Rodriguez M.A., Dang N., Smaniego F.,** ***A Phase II study of interleukin-12(IL-12) in patients with relapse non-Hodgkin lymphoma and Hodgkin disease (abstract).*** **Amercan Society of Hematology meeting, 2002.**

272. **NCCTG,** ***NCCTG trial of concomitant or sequential IL-12 in combination with rituximab in previously treated non-Hodgkin lymphoma patients.*** **2004, ASCO annual meeting, 2004.**

273. **Yoong, Y., et al.,** ***The effect of absolute lymphocyte count recovery kinetics on survival after autologous stem cell transplantation for non-Hodgkin's lymphoma.*** **Leukemia & Lymphoma, 2005. 46(9): p. 1287-94.**

274. **Solal-Celigny, P., et al.,** ***Follicular lymphoma international prognostic index.[see comment].*** **Blood, 2004. 104(5): p. 1258-65.**

275. **Ghielmini, M., et al.,** ***Single agent rituximab in patients with follicular or mantle cell lymphoma: clinical and biological factors that are predictive of response and event-free survival as well as the effect of rituximab on the immune system: a study of the Swiss Group for Clinical Cancer Research (SAKK).*** **Annals of Oncology, 2005. 16(10): p. 1675-82.**

276. **Ansell, S.M., et al.,** ***Cd4+ T-cell immune response to large B-cell non-Hodgkin's lymphoma predicts patient outcome.[see comment].*** **Journal of Clinical Oncology, 2001. 19(3): p. 720-6.**

277. **Turgeon, A.F., et al.,** ***Meta-analysis: intravenous immunoglobulin in critically ill adult patients with sepsis.*** **Annals of Internal Medicine, 2007. 146(3): p. 193-203.**

278. **Silins, S.L., et al.,** ***Asymptomatic primary Epstein-Barr virus infection occurs in the absence of blood T-cell repertoire perturbations despite high levels of systemic viral load.*** **Blood, 2001. 98(13): p. 3739-44.**

279. **Zhang, L., et al.,** ***Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy.*** **Journal of Experimental Medicine, 1999. 190(5): p. 725-32.**